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Elicitation for activation of the actinomycete genome's cryptic secondary metabolite gene clusters

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This review summarizes the recent advances in the elicitation approaches used to activate the actinomycete genome's cryptic secondary metabolite gene clusters and shows the diversity of natural products obtained by various elicitation methods up to June 2022, such as co-cultivation of actinomycetes with actinomycetes, other non-actinomycete bacteria, fungi, cell-derived components, and/or algae. Chemical elicitation and molecular elicitation as transcription factor decoys, engineering regulatory genes, the promoter replacement strategy, global regulatory genes, and reporter-guided mutant selection were also reported. For researchers interested in this field, this review serves as a valuable resource for the latest studies and references.

1. Introduction

Regular protocols used for the discovery of bacterial-based natural products often involve microorganism fermentation on different media followed by chemical and biological screening of the extracts, seeking any interesting bioactive compounds. The advent of genome-sequencing technology has enabled us to scan the substantial amount of published genome data for gene clusters encrypting the previously recognized natural products, bringing in a second golden age in drug discovery. Recently, numerous new approaches were published unlocking bacterial cryptic pathways, including studies that focus on raising transcription levels and enlivening dead genes.[1–3] Actinomycetes are unicellular, Gram-positive filamentous bacteria, belonging to the order Actinomycetales. They are broadly dispersed in nature and can be found in diverse habitats across the world. Actinomycetes are notable for producing a diverse variety of natural products with a wide range of biological activities and structurally diverse specialized metabolites.[1] Over 200 drugs based on metabolites isolated from actinomycetes are currently in clinical trials or have been approved by the FDA for the treatment of infections, immune system abnormalities and cancer chemotherapeutic and to treat emerging health threats.[2] Under conventional culture conditions, the majority of bioactive metabolites encrypted in actinomycete genomes are not activated and tendency to rediscover known secondary metabolites increases.[3] Therefore, various approaches has been devoted to eliciting the underlying gene clusters in order to discover and isolate novel lead natural products and molecular scaffolds;[4–6] such as: mixed fermentation or co-cultivation,[7] fermentation conditions alteration which known as "one strain many compounds" (OSMAC)[8] and eliciting the bacterial cells with external signals either chemically or biologically.[9,10] This review summarizes notable and successful examples of elicitation methods based on literature search from 2015 until June 2022, including changing culture conditions, modifying the medium, co-cultivating with different strains, and adding a biosynthetic precursors or epigenetic modifiers (Fig. 1). This review is complementary to the first part that was previously published.[11]

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2. Biological elicitation

Biological elicitation is one of the most actively pursued techniques to trigger the synthesis of natural products in actinomycetes.

2.1. By microbial co-cultivation

The confrontation of microorganisms in various habitat may activate cryptic gene clusters and trigger silent pathways to produce new secondary metabolites. These outcomes are based on the effective interactions between co-cultivated microorganisms.[12] For example, horizontal gene transfer, physical cell–

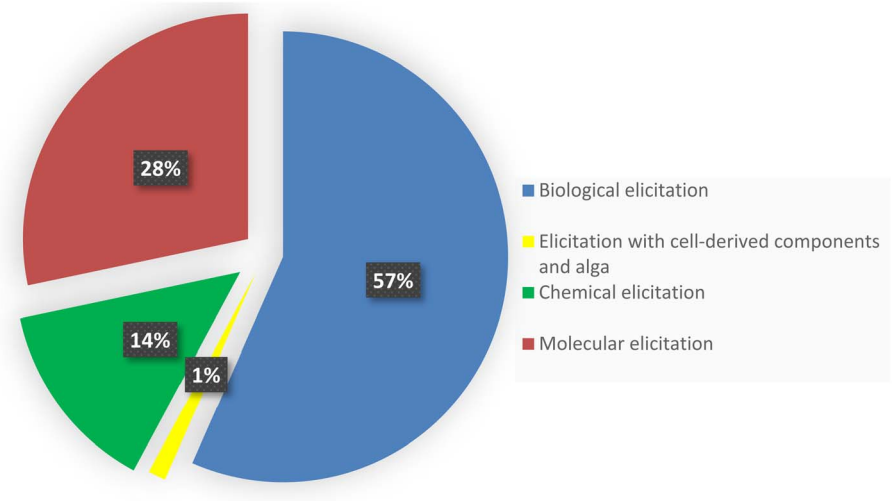

Fig. 1 Graph representing the percentage of application of different elicitation methods.

cell interactions and production of enzymes or quorum sensing molecules.[11] Co-cultivation-based elicitation can thus lead to the generation of molecules that are not synthesized in monoculture, thus aiding in the discovery of cryptic and weakly expressed secondary metabolites. However, finding suitable partners for co-cultivation remains challenging. Co-cultivation can be further categorized as (a) co-cultivation of actinomycetes with other actinomycetes, (b) co-cultivation of actinomycetes with other non-actinomycete bacteria, and (c) co-cultivation of actinomycetes with fungi. The following section describe the process, the differences and the results on each of the three categories mentioned above.

2.1.1. Co-cultivation of actinomycetes with actinomycetes (Table 1 and Fig. 2). Co-culture of two actinobacteria from the Red Sea, *Rhodococcus* sp. UR59 and *Actinokineospora spheciospongiae* strain EG49 revealed stimulation of several metabolites production which were not observed in their axenic cultures. Using biologically guided approach, angucyclines; actinosporins E (**1**), H (**2**), G (**3**), tetragulol (**4**) and the anthraquinone, capillasterquinone B (**5**) were isolated and identified. The isolated metabolites showed *in vitro* antimalarial activity against *Plasmodium falciparum*.[13]

Co-cultivation of two actinomycetes associated with marine sponge, *Actinokineospora* sp. EG49 and *Micromonospora* sp. UR56 resulted in the accumulation of five metabolites that were not traced in their independent cultures. These metabolites were isolated and identified as dimethyl phenazine-1,6-dicarboxylate (**6**), phencomycin (**7**), tubermycin (**8**), *N*-(2-hydroxyphenyl)-acetamide (**9**), *p*-anisamide (**10**). Compounds **6–8** and **10** showed high antibacterial, antibiofilm and cytotoxic activities.[14]

A change in natural product biosynthesis occurred in *Micromonospora* sp. UA17 isolated from the Red Sea Sponge *Coscinoderma mathewsi*, when co-cultivated with the mycolic acid containing *Nocardia* sp. UA 23 and *Gordonia* sp. UA19. Chlorocardicin (**11**), neocopiamycin A (**12**), and chicamycin B (**13**) were identified in the co-culture extracts, but not in the corresponding monocultures. This suggests a role for mycolic acid in the stimulation of encoded natural product biosynthesis pathways. Co-culture extracts showed antibacterial, antiparasitic and antifungal activities higher than monocultures.[15] *Streptomyces* sp. UR23 co-fermentation with a strain containing mycolic acid *Nocardia* sp. UR27 stimulating the production of a possible antitrypanosomal agents bafilomycin D (**14**) and bafilomycin A1 (**15**).[16] In addition, three new compounds identified as mirilactams (C–E) (**16–18**) were isolated in the co-culture of *Actinosynnema mirum* NBRC 14064 with *Tsukamurella pulmonis* TP-B0596.[17]

Nguyen and his colleagues found that the butenolide compounds in *Streptomyces albus* J1074 stimulated the production of avermectin (**19**) in *Streptomyces avermitilis* in an *in vivo* metabolic profiling assay with imaging mass spectrometry (IMS). This demonstrated the complex chemical interactions in streptomycetes through interspecies signals.[18]

Furthermore, the combined culture of a *Dietzia* sp. UR66 with *Saccharomonospora* sp. UR22 resulted in the production of a novel brominated oxo-indole alkaloid Saccharomonosporine A (**20**), convolutamydine F (**21**) and (S) 6-bromo-3-hydroxy-3-(1*H*-indol-3-yl) indolin-2-one (**22**) with a promising Pim-1 kinase inhibitors that mediates tumor cell growth inhibitory effect, along with other known induced metabolites; (S) 6-bromo-3-hydroxy-3-(1*H*-indol-3-yl) indolin-2-one (**22**) and nonactin (**23**).[19] Amycomicin (**24**), a modified fatty acid with an epoxide isonitrile head, was discovered to be a selective and potent inhibitor of *Staphylococcus aureus* after co-culture with *Amycolatopsis* sp. AA4 as the producing strain and *Streptomyces coelicolor* M145 as the inducing strain.[20]

In the co-culture broth of *Tsukamurella pulmonis* TPB0596 with a rare actinomycete *Micromonospora wenchangensis* HEK-797, only two new macrolactams (dracolactams A and B) were detected, whereas the axenic medium of strain HEK-797 yielded a 26-membered polyene macrolactam, which was probably the precursor of dracolactams A (**25**) and B (**26**).[21]

From another co-culture of *Rhodococcus* sp. WMMA-185 and *Micromonospora* sp. WMMB-235, a two-marine invertebrate-associated bacteria, a novel antibiotic, keyicin (**27**), was

Table 1 Comprehensive list of secondary metabolites reported upon co-cultivation of actinomycetes with actinomycetes

Interaction partners	Induced secondary	Metabolites activity	Reference
Actinokineospora spheciospongiae strain EG49 and *Rhodococcus* sp. UR59	Actinosporins E (**1**) Actinosporins H (**2**) Actinosporins G (**3**) Tetragulol (**4**) Capillasterquinone B (**5**)	Anti-malarial	13
Micromonospora sp. UR56 and *Actinokineospora* sp. EG49	1,6-Dicarboxylate (**6**) Phencomycin (**7**)	Antibacterial	14
	Tubermycin (**8**) *N*-(2-hydroxyphenyl)-acetamide (**9**) *p*-anisamide (**10**)	Cytotoxic antibiofilm	
Micromonospora sp. UA17, *Gordonia* sp. UA19, and *Nocardia* sp. UA 23	Chlorocardicin (**11**) Neocopiamycin A (**12**) Chicamycin B (**13**)	Antibacterial Antifungal Antiparasitic	15
Streptomyces sp. UR23 and *Nocardia* sp. UR27	Bafilomycin D (**14**) Bafilomycin A1 (**15**)	Antitrypanosomal agents	16
Actinosynnema mirum NBRC 14064 *Tsukamurella pulmonis* TP-B0596	Mirilactams C–E, 1–3 (**16, 17, 18**)	Macrolactams	17
Streptomyces avermitilis and *Streptomyces albus* J1074	Avermectin (**19**)	Pesticides	18
Saccharomonospora sp. UR22 and *Dietzia* sp. UR66	Saccharomonosporine A (**20**) Convolutamydine F (**21**) (S) 6-Bromo-3-hydroxy-3-(1*H*-indol-3-yl) indolin-2-one (**22**)	Antiproliferative activity	19
Streptomyces coelicolor M145 *Amycolatopsis* sp. AA4	Amycomycin (**24**)	Antibiotic activity	20
Micromonospora wenchangensis HEK-797 *Tsukamurella pulmonis* TPB0596	Dracolactams A (**25**) Dracolactams B (**26**)	Antifungal antibiotics	21
Rhodococcus sp. WMMA-185 *Micromonospora* sp. WMMB-235	Keyicin (**27**)	Selectively active against gram-positive bacteria	22
Cutibacterium avidum *Bifidobacterium longum* subsp. infantis and *Bifidobacterium bifidum*	Propionate (**28**)	It is a conjugate base of a propionic acid	23
Streptomyces nigrescens HEK616 *Tsukamurella pulmonis* TP-B0596	5-Alkyl-1,2,3,4-tetrahydroquinolines (**29**)	Novel antifungals	24
Streptomyces nigrescens HEK616 *Tsukamurella pulmonis* TP-B0596	[5,5]-Spirohemiaminals (**30**)	Broad antimicrobial activities	25
Streptomyces sp. ANAM-5 and AIAH-10	Ethyl acetate extract	Antimicrobial and anticancer activities	26
Streptomyces tendae KMC006 *Gordonia* sp. KMC005	Gordonic acid (**31**)	Weak antibacterial activity	27
Streptomyces sp. NZ-6 *Tsukamurella pulmonis* TP-B0596	Niizalactams A–C (**32–34**)	Antifungal antibiotic	28
Streptomyces cinnamoneus NBRC 13823 *Tsukamurella pulmonis*	Arcyriaflavin E (**35**)	Cytotoxic	29
Streptomyces sp. CJ-5 *Tsukamurella pulmonis* TP-B0596	Chojalactones A–C (**36–37**)	Cytotoxic	30
Streptomyces lividans TK23/pGSBC1 *Tsukamurella pulmonis* TP-B0596	Goadsporin C (**38**)	Antibiotic	31
Catenuloplanes sp. RD067331 *Tsukamurella pulmonis* TP-B0596	Catenulobactins A and B (**39, 40**)	Siderphore	32
Umezawaea sp. RD066910 *Tsukamurella pulmonis* TP-B0596	Umezawamides A and B (**41, 42**)	Anti-leukemia and antifungal activity	33
Nocardiopsis sp. FU40 (ΔApoS)	Ciromicin A (**43**)	Cytotoxicity (acute myelogenous leukemia cells)	34
Rhodococcus wratislaviensis	Ciromicin B (**44**)	Cytotoxicity (stem-like myeloid progenitor cells)	35

isolated. It had specific inhibitory effect against Gram-positive bacteria Methicillin Sensitive *Staphylococcus aureus* (MSSA) and *B. subtilis*, with MIC values of 2.5 μM and 9.9 μM, respectively. Unlike many anthracyclines, keyicin may alter fatty acid metabolism and showing antibacterial activity without causing nucleic acid damage.[22]

Cutibacterium avidum growth and production of propionate (**28**) enhanced by co-cultivation with *Bifidobacterium longum*

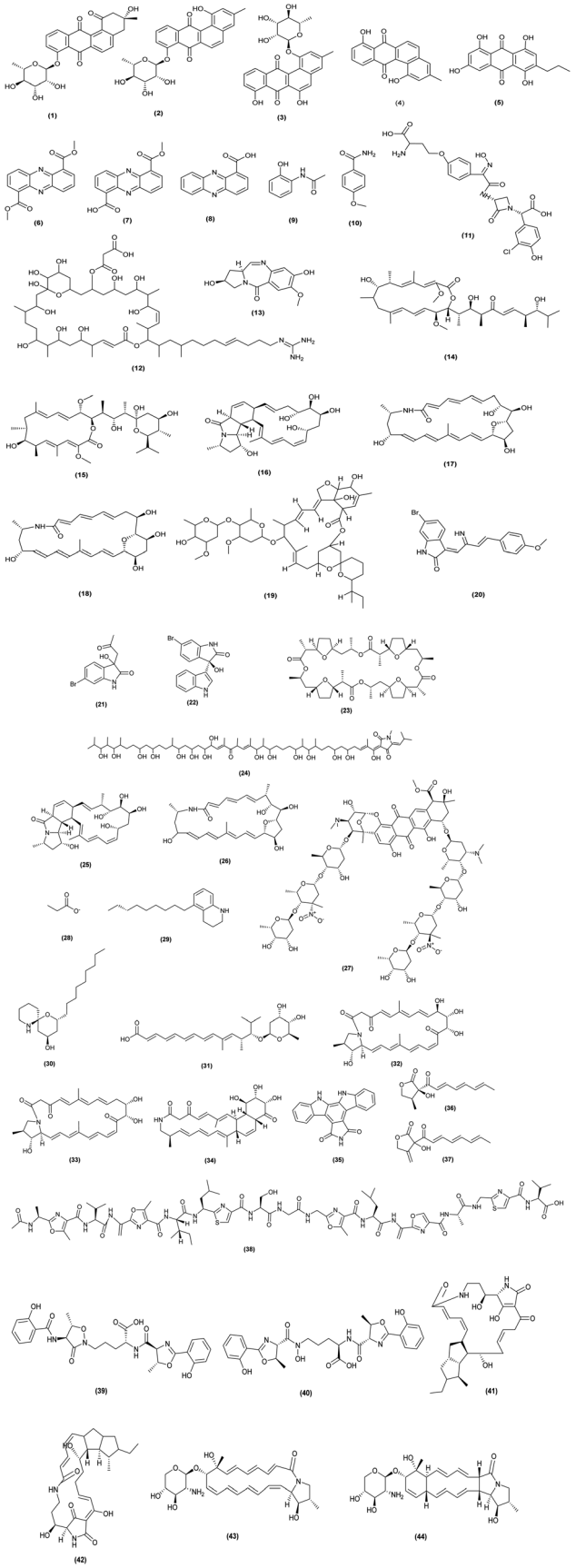

Fig. 2 Chemical structures of compounds 1–44 resulted from the co-cultivation of actinomycetes with actinomycetes. The configuration of all stereocenters in each compound is shown as given in the literature.

subsp. infantis and *Bifidobacterium bifidum* which confirmed the metabolic cross-feeding strategy.[23]

2.1.2. Co-cultivation of actinomycetes with other non-actinomycete bacteria (Table 2 and Fig. 3). Interspecies interaction between organisms living in the same environment is thought to trigger the structural variety of specialized metabolites.[11] The co-fermentation of marine strain *Streptomyces* sp. CGMCC4.7185 and *Bacillus mycoides* obtained from Nanji Island marine sediments was the first report of using a microorganism co-culture system to increase the yields of five known tryptamine derivatives. The average yields of the isolated compounds *N*-acetyltryptamine (**45**), *N*-propanoyltryptamine (**46**), bacillamide A (**47**), bacillamide B (**48**) and bacillamide C (**49**) were 14.9, 2.8, 3.0, 13.7 and 9.6 mg L^{-1}, respectively, that was better than that produced by the axenic culture.[36]

Liang *et al.* stated that co-cultivation method induced more mass characteristics than the heat-killed inducer cultures, and both approaches resulted in the initiation of major characteristics not seen with any other induction method. *N*-Carbamoyl-2-hydroxy-3-methoxybenzamide (**50**) and carbazoquinocin G (**51**), two new secondary metabolites produced through co-culturing of producer *Streptomyces* sp. RKND-216 with inducers *Alteromonas* sp. RKMC-009 and *M. smegmatis* ATCC 120515, respectively.[37]

Dentigerumycin E (**52**), a new piperazic acid-bearing cyclic peptide isolated from the co-culture of marine *Streptomyces albogriseolus* strain B24 and *Bacillus cereus* and shown anti-metastatic and antiproliferative activities against: liver, lung, stomach, breast and colorectal cancer.[38]

Co-cultivation of *Streptomyces* sp. PTY087I2 with human pathogens such as: *Bacillus subtilis*, *Pseudomonas aeruginosa,* methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*, resulted in increased production of the three antibiotics, granaticin (**53**), granatomycin D (**54**), and dihydrogranaticin B (**55**). Furthermore, biological activities against the Gram-positive human pathogens used in these study was significantly increased.[39] Increasing the use of co-culture studies to enable competitive interactions could boost metabolite synthesis and help to learn more about these microbial relationships.

2.1.3. Co-cultivation of actinomycetes with fungi (Table 3 and Fig. 4). The co-culture of *Aspergillus niger* and *Mycobacterium smegmatis* led to the production of a pigment by *A. niger* as well as an enhance in the extract's cytotoxic activity against human prostate cancer cells. An examination of natural products in the co-culture broth extract revealed that the increase in cytotoxic activity was driven by the development of malformin C (**60**), as well as the production of TMC-256A1, desmethylkotanin, and aurasperone C under co-culture conditions.[44]

In *Streptomyces natalensis* HW-2 fermentation, a fungal elicitor from *Penicillium chrysogenum* AS 3.5163 showed an inductive effect on the production of natamycin (**61**). It was first observed that the fungal elicitor altered transcriptional levels in *S. natalensis* HW-2. The main conclusion is that the fungal elicitor increases the amount of precursor and changes the expression of natamycin related genes and secondary metabolic regulators.[45]

Co-cultivation of *Bionectria* sp., obtained from the seeds of the tropical plant *Raphia taedigera* with *Streptomyces lividans*

Table 2 Comprehensive list of secondary metabolites reported upon co-cultivation of actinomycetes with other non-actinomycete bacteria

Interaction partners	Induced secondary	Metabolites activity	Reference
Streptomyces sp. CGMCC4.7185 *Bacillus mycoides*	Tryptamine (**45**, **46**) Bacillamides (**47**, **48**, **49**)	Algicidal	36
Streptomyces sp. RKND-216 *Alteromonas* sp. RKMC-009	*N*-Carbamoyl-2-hydroxy-3-methoxybenzamide (**50**)	Antimicrobial Cytotoxic activity	37
Streptomyces sp. RKND-216 *M. smegmatis* ATCC 120515	Carbazoquinocin G (**51**)	Antimicrobial Cytotoxic activity	37
Streptomyces albogriseolus strain B24 *Bacillus cereus*	Dentigerumycin E (**52**)	Antiproliferative Antimetastatic	38
Streptomyces sp. Strain PTY087I2 methicillin-resistant *Staphylococcus aureus* (MRSA)	Granaticin (**53**) Granatomycin D (**54**) Dihydrogranaticin B (**55**)	Increased antibacterial activity	39
Streptomyces coelicolor *Myxococcus xanthus* DK1622	Actinorhodin (**56**)	A benzoisochromanequinone dimer polyketide antibiotic	40
Streptomyces coelicolor A3 *E. coli* C600	Undecylprodigiosin (**57**)	Immunosuppressive Antitumor	41
Streptomyces sp. MA37 *Pseudomonas* sp.	Be-13793c (**58**)	Anti-cancer activity	42
Streptosporangium sp. KDCAGE35 *Bacillus* sp. Strain KDCAGE13	Funisamine (**59**)		43

yielded two novel *o*-aminobenzoic acid derivatives, bionectriamines A (**62**) and B (**63**), along with two other previously reported tris(2,4-di-*tert*-butylphenyl) phosphate (**64**) and 6,8-dihydroxyisocoumarin-3-carboxylic acid (**65**). None of the reported compounds were found in axenic fungal or bacterial cultures, showing that co-cultivation with bacteria activated silent biogenetic gene clusters.[46] Amphotericin B (**66**), a well-known antifungal compound resulted from the co-cultivation of *Streptomyces albospinus* RLe7 and *Coniochaeta* sp. FLe4, endophytes isolated from the roots of the Brazilian medicinal plant *Lychnophora ericoides*. Amphotericin B was identified as one of the substances responsible for antifungal action as well as the induction of a red-pigmented fungal phenotype.[47]

A series of cytochalasans (rosellichalasin (**67**) and five aspochalasins (**68–72**)) were produced as a result of the co-culture of *Aspergillus flavipes* and *Streptomyces* sp. which isolated from the same marine sediment. These cytochalasans were reported to be formed by *A. flavipes*, and they were able to have cytotoxic activity against *Streptomyces* sp. at a wide range of concentrations while having no effect on the producer, favoring the producer in competition.[48] The secondary metabolism of the filamentous model microorganisms *Streptomyces coelicolor* and *Aspergillus niger* is greatly influenced by their co-cultivation. *A. niger* formed the cyclic dipeptide cyclo (Phe–Tyr) (**73**), cyclo (Phe–Phe) (**74**), phenylacetic acid (**75**), furan-2-carboxylic acid (**76**) and 2-hydroxyphenylacetic acid (**77**) in reaction to *S. coelicolor*, according to NMR-based metabolomics combined with multivariate data analysis.[49]

3. Elicitation with cell-derived components and alga (Fig. 4)

Many studies have been conducted to induce antibiotic encoding biosynthetic genes in actinomycetes by chemical and biological elicitors, which lead to production of numerous valuable bioactive metabolites based on the whole genome results. Mohammadipanah *et al.* revealed that the supernatant of *Pseudomonas aeruginosa* UTMC 1404 culture could act as stimuli to induce antibacterial synthetic pathways in *Promicromonospora kermanensis* DSM 45485. The eliciting agents in *P. aeruginosa* culture cell filtrate were resistant to detergent, acidic, and basic conditions and had an amphipathic character.[58]

Mixed fermentation of *Nocardia bhagyanarayanae* I-27 and green microalga *Tetradesmus obliquus* AARL G022 yielded essential Ω3 fatty acids, including eicosapentaenoic acid (**97**) and α-linolenic acid (**98**).[59] Eicosapentaenoic acid granted by the European Commission as Orphan designation for the treatment of familial adenomatous polyposis.

4. Chemical elicitation (Table 4 and Fig. 5)

Antibiotics at sub-inhibitory concentrations (SIC) have been demonstrated to alter gene expression at the transcriptional level, influencing 5–10% of all transcripts. Many phenotypic changes have been seen in the presence of sub-inhibitory antibiotic concentrations, including biofilm formation and enhanced bacterial motility. Antibiotic at SICs are being employed to trigger the production of cryptic natural compounds.[60] Lincomycin at 1/10 of its MIC significantly boosted the expression of the pathway-specific regulatory gene *act*II-ORF4 in the blue-pigmented antibiotic actinorhodin (ACT) (**99**) biosynthetic gene cluster, resulting in ACT overproduction in *Streptomyces coelicolor* A3(2).[61]

Interestingly, it was found that addition of a SIC (0.1 μM) of triclosan to the culture media of *S. coelicolor* A3 (2) strain 1147 (wild-type) increased actinorhodin (**99**) synthesis by 5.2-fold. Triclosan had a positive effect on actinorhodin even in the high-producing *S. coelicolor* strain KO-1130 (rpoB), elevating titers by

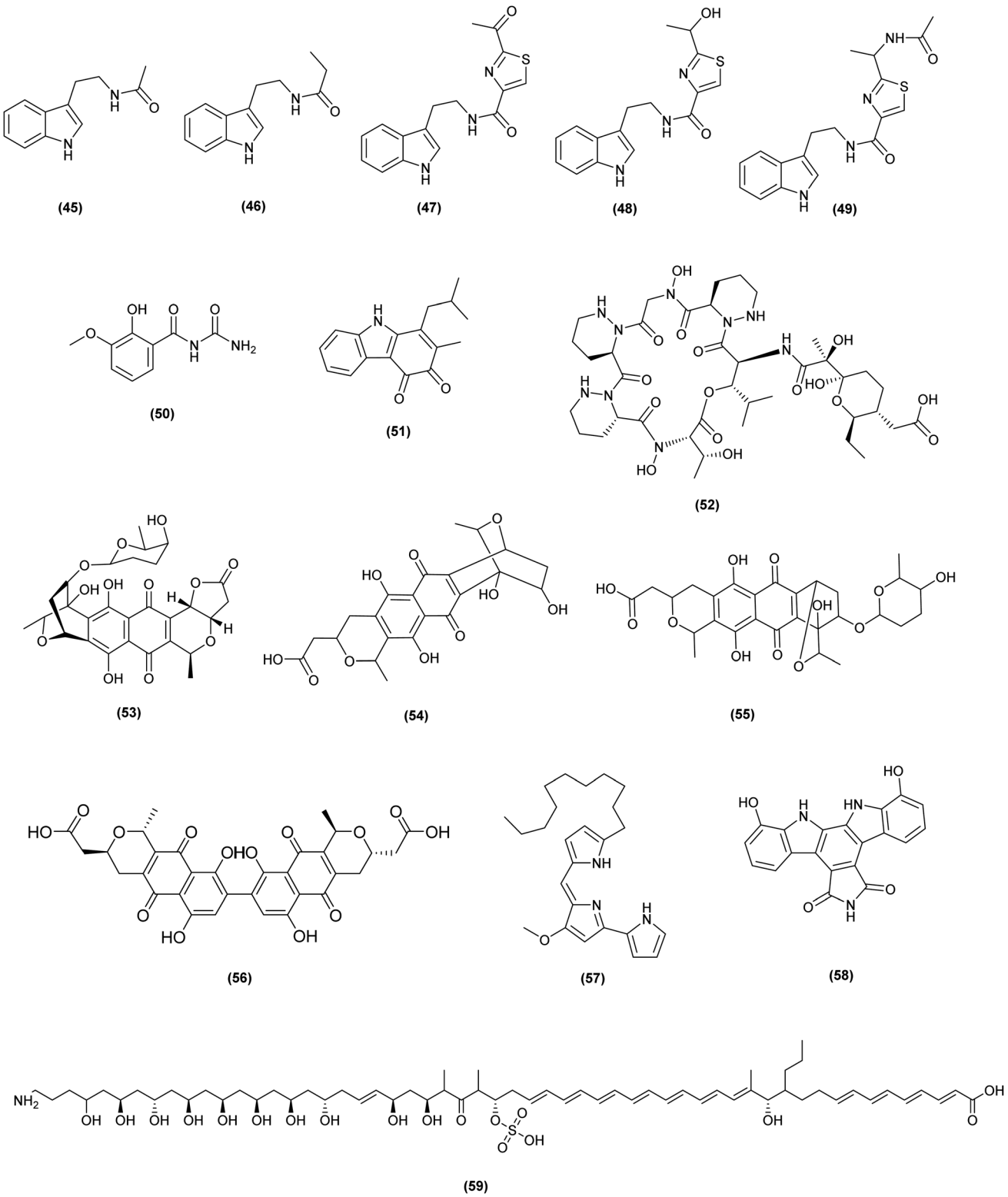

Fig. 3 Chemical structures of compounds 45–59 reported upon co-cultivation of actinomycetes with other non-actinomycete bacteria. The configuration of all stereocenters in each compound is shown as given in the literature.

82%. Moreover, addition of a SIC (1 μM) of triclosan to *Streptomyces albus* industrial strain KO-606 increased salinomycin (**100**) production by 40%, achieving 30.4 g L^{-1} of salinomycin titer. At subinhibitory concentrations of 0.3 g ml^{-1} (1/30 of its MIC), chloramphenicol (ribosome-targeting drug) increased the production of nonribosomal peptide antibiotic (actinomycin D) (**101**) in *S. antibioticus* strain KO-1164 by 1.6-fold CDA by *S. coelicolor* A3(2), presumably *via* increasing the intracellular

Table 3 Comprehensive list of secondary metabolites reported upon co-cultivation of actinomycetes with fungi

Interaction partners	Induced secondary	Metabolites activity	Reference
Mycobacterium smegmatis *Aspergillus niger*	Malformin C (**60**)	Increase in cytotoxic activity	44
Streptomyces natalensis *Penicillium chrysogenum*	Natamycin (**61**)	Polyene macrolide polyketide antibiotics	45
Streptomyces lividans	Bionectriamines A (**62**) Bionectriamines B (**63**)		46
Bionectria sp.	Tris(2,4-di-*tert*-butylphenyl) phosphate (**64**) 6,8-Dihydroxyisocoumarin-3-carboxylic acid (**65**)		
Streptomyces albospinus RLe7 *Coniochaeta* sp. FLe4	Amphotericin B (**66**)	Antifungal activity Induction of red pigmented fungal phenotype	47
Aspergillus flavipes *Streptomyces* sp.	Cytochalasans (**67–72**)	Cytotoxic effects against Streptomyces sp.	48
Streptomyces coelicolor *Aspergillus niger*	Cyclo (Phe–Tyr) (**73**) Cyclo (Phe–Phe) (**74**) Phenylacetic acid (**75**) Furan-2-carboxylic acid (**76**) 2-Hydroxyphenylacetic acid (**77**)		49
Streptomyces rochei MB037 *Rhinocladiella similis* 35	Borrelidin (**78**) Borrelidins F (**79**), J (**80**) and K (**81**) 7-Methoxy-2,3-dimethylchromone-4-one (**82**)	Antibacterial activity	50
Streptomyces leeuwenhoekii C34 *Aspergillus fumigatus* MR2012	Luteoride D (**83**) Pseurotin G (**84**) Terezine D (**85**) 11-*O*-methylpseurotin A (**86**)		51
Streptomyces leeuwenhoekii C58 *Aspergillus fumigatus* MR2012	Chaxapeptin Pentalenic acid (**87**)	Inhibit the human lung cancer cell line A549	51
Streptomyces albospinus RLe7 *Phomopsis* sp. FLe6	Djalonensone (**88**) 2,5-Furandimethanol (**89**)		52
Streptomyces piomogenus AS63D *Aspergillus niger*	Penicisteroid C (**90**)	Antimicrobial Cytotoxic activities	53
Streptomyces sp. CMB-M0423 *Aspergillus* sp. CMB-StM0423	Heronapyrrole B (**91**)	Fungistatic	54
Aspergillus fumigatus *Streptomyces rapamycinicus*	Fumigermin (**92**)	A reversible germination inhibitor	55
Trichoderma sp. (307)	(3*R*, 7*R*)-7-hydroxy-de-*O*-methyllasiodiplodin (**93**)	Potent α-glucosidase inhibitory activity	6
Acinetobacter johnsonii (B2)	(3*R*)-5-oxo-de-*O*-Methyllasiodiplodin (**94**)		
Streptomyces coelicolor A3 (2) *Aspergillus niger* N402	Gtri-02 (**95**)		56
Actinomycete WAC 2288 *Cryptococcus neoformans*	Ibomycin (**96**)	Anticryptococcus activity	57

amino acid pool size.[62] From GYM agar plates containing various concentrations of streptomycin and paromomycin at doses of 1.5- to 10-fold greater than their respective MICs, 307 spontaneous antibiotic-resistant mutants of *Streptomyces diastatochromogenes* strain 1628 were identified. When streptomycin and paromomycin were used as screening antibiotics, the yields of toyocamycin (**102**), tetramycin A (**103**), tetramycin P (**104**) and tetrin B (**105**) were 244, 680, 583, and 569 mg L^{-1}, respectively, which were 4.1-, 7.8-, 5.1-, and 13- fold higher than the yields of the wild-type strain.[63]

To uncover cryptic, bioactive metabolites, bioactivity assays are coupled with high-throughput elicitor screening (HiTES). The use of this technique in *Saccharopolyspora cebuensis*, with inhibition of *Escherichia coli* growth as a read-out, resulted in the production of cebulantin (**106**), a new lanthipeptide, induced by the clinical drugs furosemide and fenofibrate.[64]

HiTES was used successfully to awaken a cryptic gene cluster in *Streptomyces albus* J1074. The cytotoxins etoposide and ivermectin were shown to be powerful inducers, allowing identifying and isolating 14 new small chemical products from the chosen cluster. One of these metabolites is surugamide I (**107**), a strong cathepsin B inhibitor implicated in cancer, while a novel acyl-surugamide A (**108**) had good antifungal activity.[65] The production of the anthraquinones aloesaponarin II (109) and hypogeamicin B (110) from the actinomycetes *Micromonospora* sp. BBHARD22 and *Nonomuraea* sp. BBHARD23,

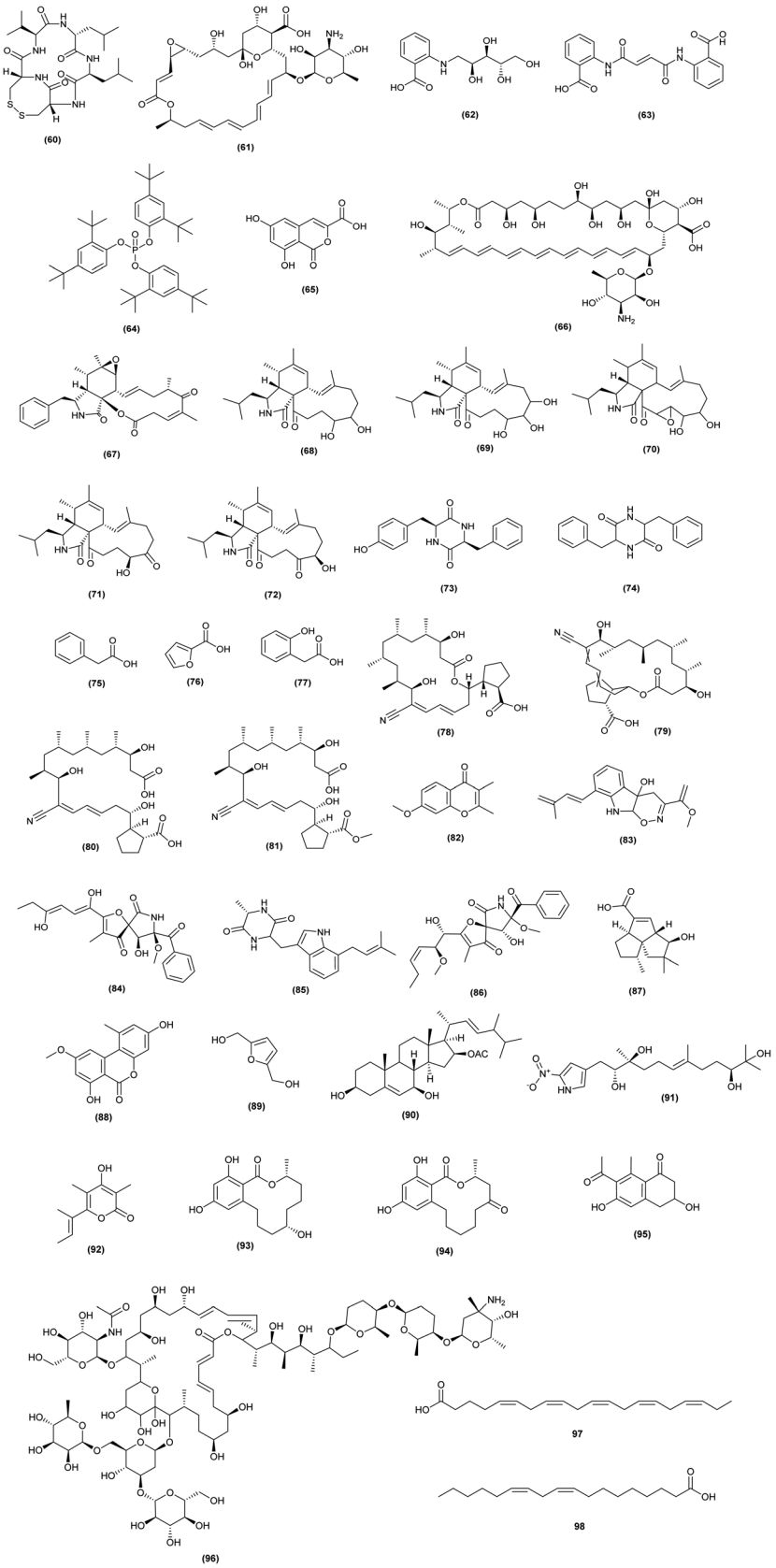

Fig. 4 Chemical structures of compounds **60–96** reported upon co-cultivation of actinomycetes with fungi. Cytochalasans: rosellichalasin (**67**), 19,20-dihydro-aspochalasin D (**68**), aspochalasin E (**69**), aspochalasin H (**70**), aspochalasin M (**71**), aspochalasin P (**72**); chemical structures of compounds **97** and **98** reported upon co-cultivation of actinomycetes with alga. The configuration of all stereocenters in each compound is shown as given in the literature.

Table 4 Comprehensive list of secondary metabolites reported upon chemical elicitation

Elicitor	Strain	Secondary metabolites Induced	Mechanism of elicitation	References
Lincomycin	*Streptomyces coelicolor* A3	Actinorhodin (**99**) antibiotic	At 1/10 of its MIC, lincomycin significantly boosted the expression of the pathway-specific regulatory gene actII-ORF4 in the blue-pigmented antibiotic actinorhodin (ACT) biosynthetic gene cluster, resulting in ACT overproduction	61
Triclosan	*S. coelicolor* A3(2) strain 1147 (wild-type)	Actinorhodin (**99**)		62
Triclosan	*Streptomyces albus* industrial strain KO-606	Salinomycin (**100**)		
Chloramphenicol	*S. antibioticus* strain KO-1164	Nonribosomal peptide (NRP) antibiotics Actinomycin D (**101**), calcium-dependent antibiotic (CDA), and piperidamycin)	Chloramphenicol raised amino acid pool sizes 1.5 to 6-fold, boosting CDA and actinomycin production	
Streptomycin and paromomycin	*Streptomyces diastatochromogenes 1628*	Toyocamycin **(102)** Tetramycin A (**103)** Tetramycin P (**104)** Tetrin B (**105)**	Ribosome engineering strategies for activating genes for toyocamycin biosynthesis	63
Furosemide fenofibrate	*Saccharopolyspora cebuensis*	Cebulantin (**106**) (Inhibitory effect on vibrio strains)	ceb, a class I lanthipeptide BGC	64
Etoposide and ivermectin	*Streptomyces albus J1074*	Surugamide I (**107**) inhibit a cysteine protease implicated in cancer Acyl-surugamide A (**108**) a novel antifungal	Chemogenetic high-throughput screening approach ("HiTES") to discover small molecule elicitors of silent biosynthetic gene clusters	65
Scandium	*Micromonospora* sp. *BBHARD22* *Nonomuraea* sp. *BBHARD23*	Aloesaponarin II (**109**) Hypogeamicin B (**110**)		
Streptomycin	*Microbispora* sp. *BCCAGE54*	Tetarimycin B (**111**)	Identified through the heterologous production of an environmental-derived type II polyketide synthase (PKS) gene cluster	43
Histone deacetylase inhibitors (hdais)(valproic acid)	*Promicromonospora kermanensis* DSM 45485		Antibacterial activity	50
Trimethoprim	*Burkholderia thailandensis*	Acybolins A–I and bactobolin (**112-115**)	Antibiotic	66
Ferric ion	*Streptomyces* sp. *FXJ1.172*	Antibacterial cyclodepsipeptide, named NC-1 (**116**)	Lead to the activation of an uncharacterized cyclodepsipeptide, NC-1	67
Rifampicin	*Streptomyces somaliensis SCSIO ZH66*	Fredericamycin A (**117**)	Using ribosome engineering and response surface methodology	68
Cl-ARC	*Streptomyces ghanaensis* ATCC 14672 *Streptomyces hygroscopicus* ATCC 53653 *Streptomyces* sp. WAC0256	Oxohygrolidin (**118**) 9-Methylstreptimidone (**119**) Cyclotetralactone dynactin Nonactin (**23**)	Elevate the expression of cryptic biosynthetic genes	69

isolated from hypogean cave, increased six-fold under scandium metal exposure conditions. Tetarimycins (**111**) production from *Microbispora* sp. BCCAGE54 was increased thirty-threefold when exposed to streptomycin.[43]

5. Molecular elicitation

In addition to the cultivation-dependent elicitation techniques previously discussed, a review of metabolic product

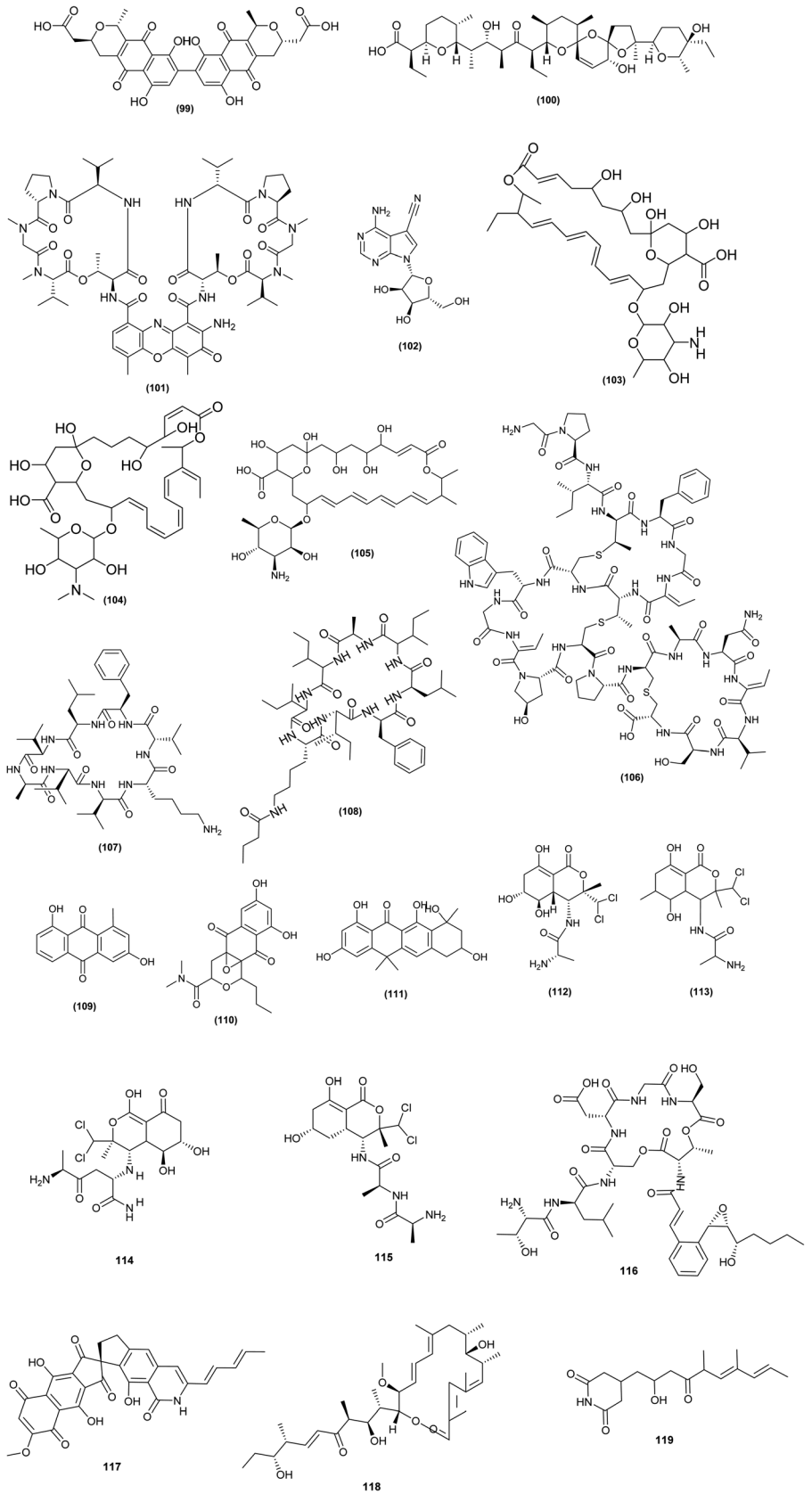

Fig. 5 Chemical structures of compounds **99–119** reported upon chemical elicitation. Bactobolins: bactobolin (**106**), bactobolin A (**107**), bactobolin B (**108**), bactobolin C (**109**).

Table 5 Comprehensive list of secondary metabolites reported upon molecular elicitation with transcription factor decoys, overexpression of regulatory genes, promoter replacement strategy, reporter-guided mutant selection and global regulatory genes

Elicitor	Strain	Secondary metabolites Induced	Mechanism of elicitation	References
Phosphopantheteine transferases (PPtase)	*Streptomyces alboniger* NRRL B-1832	Puromycin A (**120**) Puromycin B (**121**) Puromycin C (**122**)	Overexpression of PPtases affects secondary metabolism since it regulates primary metabolism, which causes physiological changes and changes in metabolic flux	72
Avenolide	*Streptomyces avermitilis*	Avermectin (pesticides) (**123**)		73
TFDs, Rssp8M28I can activate butyrolactol A and Rssp7M24I can activate oxazolepoxidomycin A	*Streptomyces* sp. F-5635 (ssp8) *Streptomyces* sp. F-4335 (ssp7)	Butyrolactol A (**124**) Oxazolepoxidomycin A (**125**)	The presence of TFDs in the cell can bind to repressors, reducing the interaction between repressors and promoters and resulting in the activation of the silenced genes	74
Overexpression of regulatory genes				
SARP-type regulator gene papR2	*Streptomyces* sp. SHP22-7	Amicetin (**126**) Plicacetin (**127**)	Amicetin/plicacetin gene cluster activation induced by PapR2	75
SigA	*Corynebacterium glutamicum*	β-carotene (**128**) Bisanhydrobacterioruberin (BABR) (**129**)	In the stationary development phase, overexpression of sigA caused a 2-fold increase in the accumulation of the native carotenoid decaprenoxanthin	76
Promoter replacement strategy				
pCRISPR-Cas9-LigD and pCRISPomyces-2 sgRNA	*Streptomyces* sp. WAC5374, WAC8241 and 6273	Thiolactomycin (**130**) Amicetin (**126**) Phenanthroviridin (**131**) 5-Chloro-3-formylindole (**132**)	ligD, an enzyme necessary for NHEJ, and a homology template are both present in the plasmid	4
CRISPR-Cas9 technique	*S. roseosporus* NRRL 15998	Auroramycin (**133**)	Utilising a CRISPR-Cas9-based knock-in of KasOp* promoter's silent type I PKS BGC	78
	Streptomyces viridochromogenes	Pentangular type II polyketide (**134**)	Effective and precise promoter cassette introduction using CRISPR-Cas9 promotes expression of biosynthetic genes and causes the creation of distinctive metabolites that are not seen in the parent strain	83
	S. roseosporus	Macrolactam (**135**) Photocyclized alteramide A (**136**) FR-90098 9 (**137**)		83
Global regulatory genes				
whiB-like (wbl) regulatory genes	*Streptomyces somaliensis* SCSIO ZH66	Violapyrone B (**138**) Violapyrone A (**139**) Violapyrone J (**140**) Violapyrone C (**141**) Violapyrone H (**142**)	Play significant roles in secondary metabolism and morphological differentiation	79
ΔadpA	*Streptomyces ansochromogene*	Oviedomycin (**143**)	adpa, a global regulatory gene, is disrupted to activate a cryptic oviedomycin gene cluster (pks7)	5
adpAa gene inactivation or abrC3 global regulatory gene overexpression	*Streptomyces argillaceus* ATCC 12956	Antimycin A1a (**144**) Antimycin A1b (**145**) Antimycin A2a (**146**) Antimycin A2b (**147**) Antimycin A3a (**148**) Antimycin A3b (**149**) Antimycin A4a (**150**)	Regulatory elements that respond to butyrolactone hormone	80

Table 5 (*Contd.*)

Elicitor	Strain	Secondary metabolites Induced	Mechanism of elicitation	References
		Antimycin A4b (**151**) Carotenoids (leprotene) (**152**) 3-Hydroxyleprotene (**153**) 3,3′-dihydroxyleprotene (**154**) β-isorenieratene (**155**) Germicidins B (**156**) Germicidins C (**157**) Desferrioxamine B (**158**)		
Reporter-guided mutant selection				
XylE-neo cassette + the promoter of ccaR gene (XylE, a catecholase that dyes colonies brown when catechol is present, was fused to neo, a gene that codes for kanamycin resistance + ccaR gene, a transcriptional activator of clavulanic acid)	*Streptomyces venezuelae* ISP5230 and *Streptomyces* sp. PGA64	Jadomycin B (**159**) Gaudimycin D (**160**) Gaudimycin E (**161**)	Streptomyces spp. reporter-guided mutant selection. The strain is mutagenized using UV light after a double reporter construct called xylE-neo is put under the control of a selected quiet promoter	81
Mutagenesis by Tn insertion in conjunction with genetic reporters	*Streptomyces albus* J1074	Antimycins (**144–151**) and candicidins (**162**)	The XNR 3174 gene, which encodes an unidentified transcriptional regulator, was shown to be the site of transposon insertion in the most well-known strain of S. albus, ATGSal2P2:TN14. Avenolide-like substance butenolide 4 was discovered to be produced by the mutant strain	82
Transposon (Tn) mutagenesis	*Streptomyces coelicolor*	Tripyrrole antibiotic: Undecylprodigiosin (**163**) and streptorubin B (RED (**164**))	A hyperactive transposase-based Tn5 transposition system trigger's 724 mutants involving 365 genes, includes 17 genes in the RED biosynthetic gene cluster	84

identification techniques for microorganisms with silent gene clusters discovered through genome mining has been undertaken. Another way to explore the actinomycetes' hidden potential is to mine microbial genomes for silent gene clusters, characterize the cryptic products using gene-knockout experiments, and combine these processes with analytical techniques like MALDI-TOF imaging and HPLC-MS.[70]

5.1. Transcription factor decoys (TFD) (Table 5 and Fig. 6)

Many methods have been devised to fully activate the untapped biosynthetic gene cluster (BGC), either by cloning and expressing the whole biosynthetic gene cluster (BGCs) or by directly modifying regulators in native hosts. Transcription factor decoys are DNA molecules that are created to interfere with gene regulation and may cause both the deactivation of a target BGC that is naturally active as well as the de-repression of a target BGC that is silent.[71]

Puromycin A (**120**), a potent cytotoxic agent, along with two new derivatives (puromycin B and C) (**121**,**122**) were identified from *Streptomyces alboniger* NRRL B-1832 by enhancing the phosphopantetheinylation of carrier proteins. To activate the puromycin cryptic/silenced biosynthetic pathway, two broad-selective phosphopantetheinyl transferase (PPtase) genes from *Bacillus subtilis* and *S. verticillus* were conjugated into the *S. alboniger* NRRL B-1832 strain.[72] By altering the expression of a novel TetR-family regulator and its target gene product, it was possible to increase the production of the anthelmintic avermectin in *Streptomyces avermitilis*. In order to promote the production of avermectin (**123**) and morphological differentiation in *S. avermitilis*, Niu *et al.* 2016 found SAV3619, a TetR-family transcriptional regulator named AveT, to be an activator.

Transcription Factor Decoys (TFD) activation efficiently activated BGCs from *Streptomyces* sp. F-5635 (ssp8) (a) and *Streptomyces* sp. F-4335 (ssp7), resulting in the isolation of

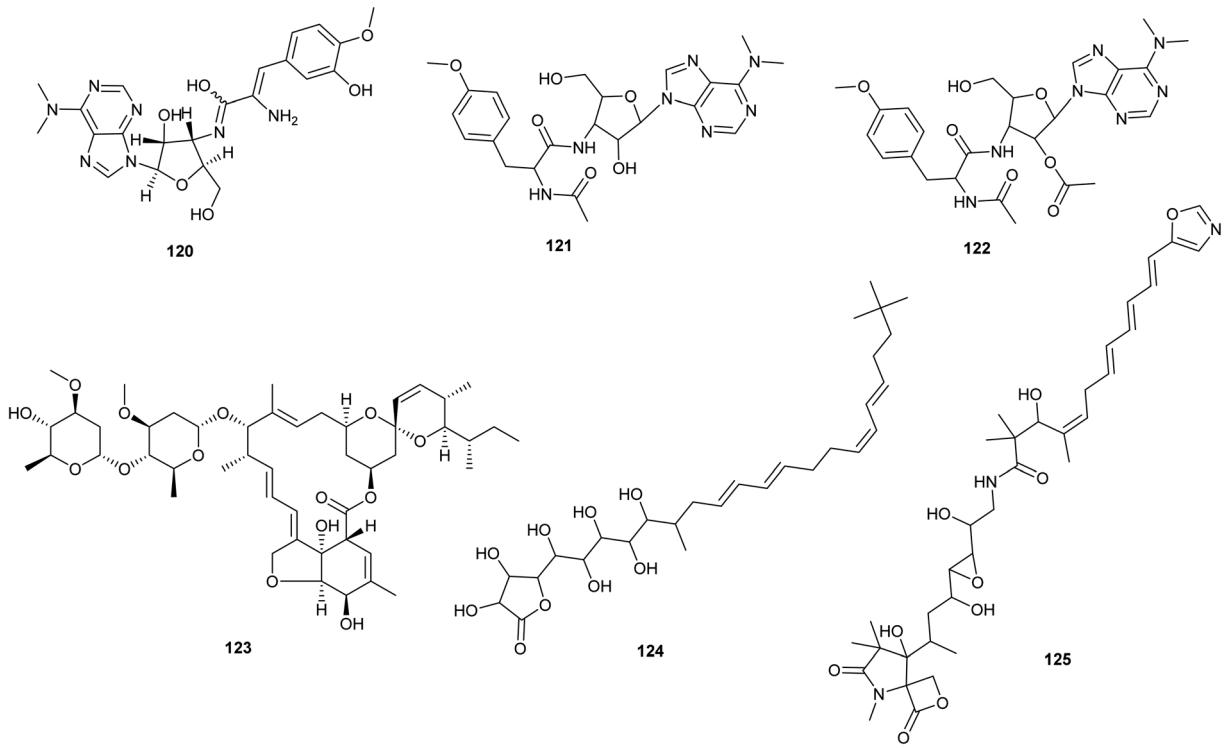

Fig. 6 Chemical structures of compounds 120–125 reported upon molecular elicitation with transcription factor decoys.

butyrolactol A (**124**), a wide antifungal, and a new chemical, oxazolepoxidomycin A (**125**).[74]

5.2. Overexpression of regulatory genes (Table 5 and Fig. 7)

The streptomyces antibiotic regulatory protein (SARP) that regulates PapR2 expression in *Streptomyces lividans* or *Streptomyces* sp. SHP22-7 leads to a significantly improved antibiotic BGC such as: red amicetin (**126**) and plicacetin (**127**) (broad-spectrum antibacterial and antiviral disaccharide pyrimidine nucleoside antibiotics), respectively.[75] β-carotene (**128**) and the non-native bisanhydrobacterioruberin (**129**) production were stimulated by overexpression of the principal sigma factor gene sigA from *Corynebacterium glutamicum*.[76]

5.3. Promoter replacement strategy (Fig. 7)

As a selective and effective method for gene editing, CRISPR/Cas9 knocks in promoter cassettes to cause the expression of biosynthetic genes. As a result, more strain collections were mined in search of new antibiotics.[77] (Culp *et al.* 2019). employed CRISPR-Cas9 genome engineering to inactivate the genes producing two of the most prevalent antibiotics, streptothricin and streptomycin, in 11 actinomycete strains. As a result, the modified strain WAC6273 orf17 was used to isolate the uncommon antibiotic amicetin and its new derivatives. Besides, the antibacterial substances thiolactomycin (**130**), phenanthroviridin (**131**), and 5-chloro-3-formylindole (**132**) were discovered utilizing activity-guided purification from two distinct WAC5374 and WAC8241 ΔstrI altered strains.[4]

In *Streptomyces roseosporus* NRRL 15998, Lim *et al.* 2018 found auroramycin (**133**), a strong glycosylated polyene macrolactam antibiotic, by inducing the expression of a 95 kb BGC utilizing a 97 bp kasO* promoter.

5.4. Global regulatory gene (Table 5 and Fig. 8)

Violapyrone B (VLP B) (**138**), a pyrone chemical, was identified when the whiB-like (wbl A50) regulatory gene from *S. somaliensis* SCSIO ZH66, a deep-sea-derived strain, was rendered inactive. Further investigation led to the characterization of the VLP biosynthetic gene cluster and isolation of the VLPs analogues named A (**139**), J (**140**), C (**141**) and H (**142**) as a result of inactivation of vioB.[79]

Global regulatory genes, such as *adpa*, which is the global regulatory gene in several streptomyces species, are activated by genetic manipulation, which results in the activation of cryptic gene clusters. *Adpa* was disrupted in *S. ansochromogenes*, resulting in the development of a mutant whose fermented extract displayed inhibitory zones against Gram-positive bacteria (*S. aureus*, *B. subtilis* and *B. cereus*) in addition to cytotoxic activities which done not occur in the wild type strain which indicate that disruption of *adpa* gene resulted in new metabolites. Chromatographic analysis revealed the presence of the new metabolite, oviedomycin (**143**) in Δ*adpa* fermented extract and absence of nikkomycin traditional produced by the wild type strain *adp*a.[5]

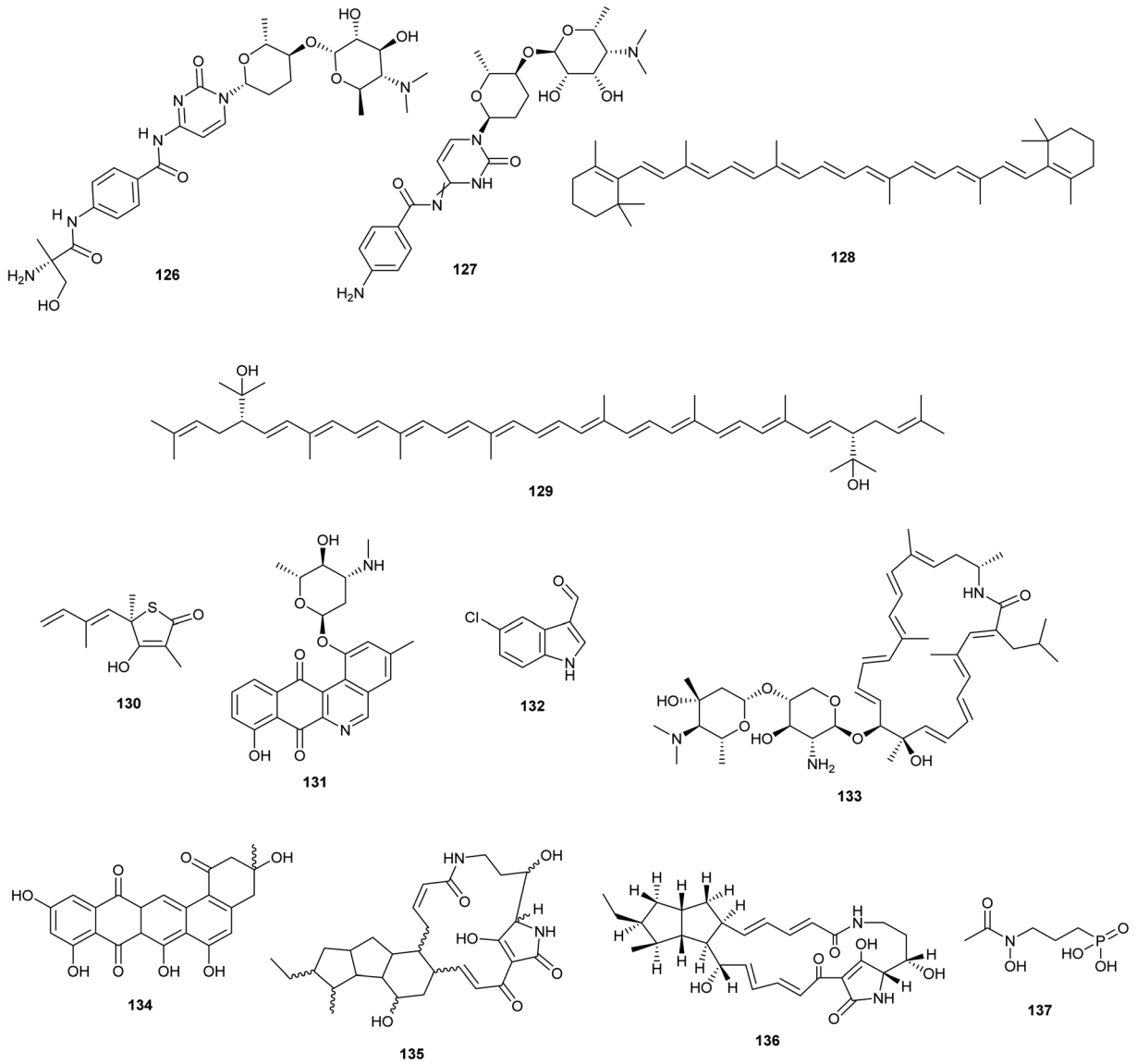

Fig. 7 Chemical structures of compounds reported upon molecular elicitation with overexpression of regulatory genes (126–129) and Promoter Replacement Strategy (130–137).

Becerril *et al.* adopted four different approaches to activate BGCs in *S. argillaceus* and isolate encoded compounds; first, they changed culture conditions of *S. argillaceus* which lead to the production of antimycins (**144–151**) as a result of the encoded gene cluster 27 being activated. Additionally, they activated the cryptic gene cluster 26 when they expressed it in a heterologous host, which led to the isolation of three new carotenoids (**152–155**). Furthermore, the discovery of novel secondary metabolites is greatly aided by the global regulatory genes. Herein, the authors inactivated the global regulatory gene *adqa* resulted in identification of two compounds; germicidins B (**156**) and C (**157**) isolated as mixture and decreased production of mithromycin. On the other hand, they overexpressed the global regulatory gene abrc3 in *S. argillaceus* resulted in the isolation of desferrioxamine (**158**) (Becerril *et al.* 2018).

5.5. Reporter-guided mutant selection (Table 5 and Fig. 9)

Reporter-guided mutant selection is one of the most recent approaches (RGMS) for activating silent gene clusters in native producers. It is an efficient and generally applicable technique, in which reporters are employed to aid in the selection of target over-expressing mutants that are then tested for the desired phenotype utilizing reporter gene expression.[81] This approach is predicated on the straightforward idea that the yield of the final metabolite is proportional to the degree of expression of the concerned biosynthetic gene cluster.[82] Guo *et al.* adopted the RGMS approach in activating two angucycline-type gene clusters that were silent in two strains of *Streptomyces* in standard laboratory conditions. In this report, authors reactivated jadomycin biosynthesis in *Streptomyces venezuelae* ISP5230 lead to isolation of jadomycin B (**159**). In addition, they activated

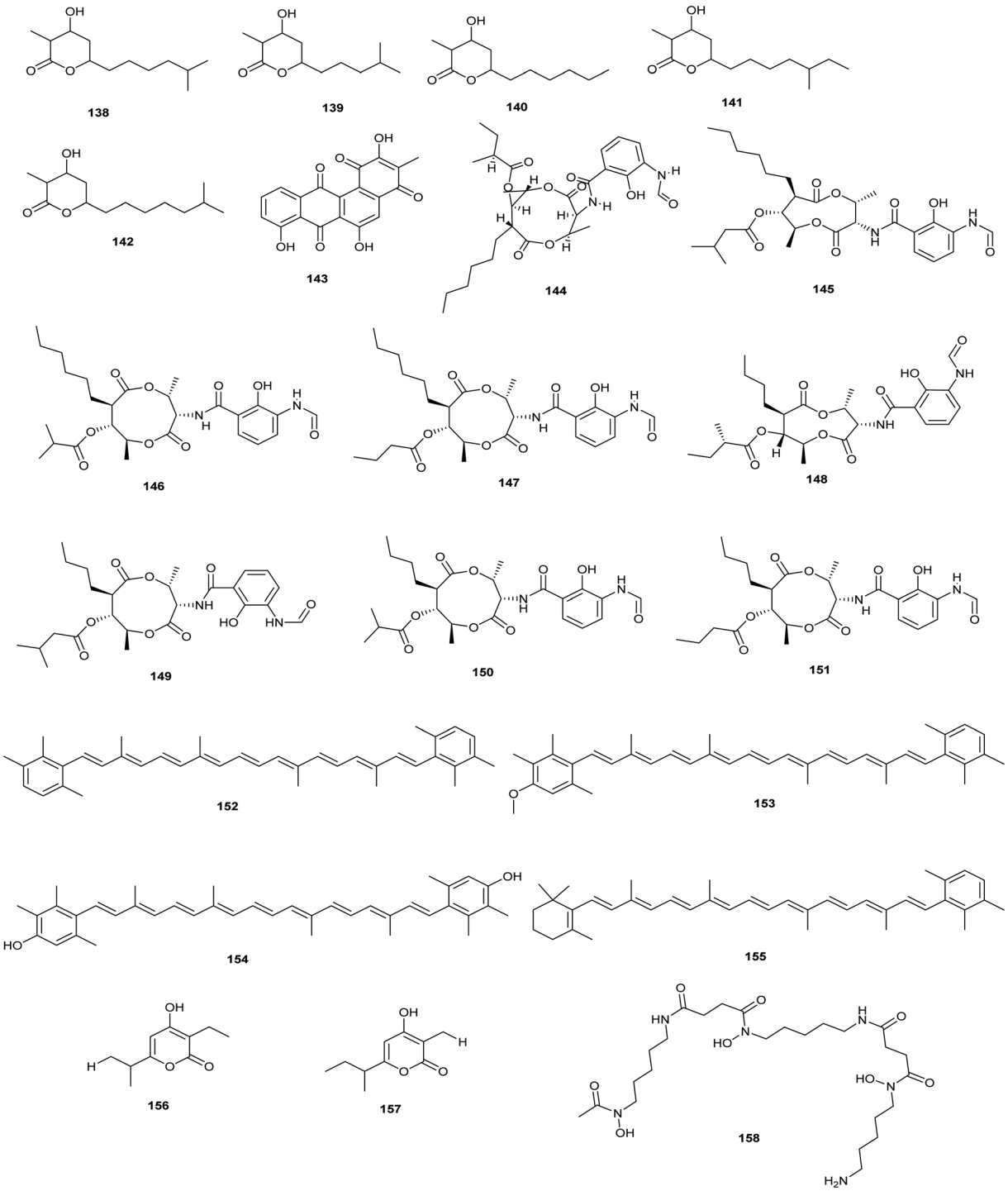

Fig. 8 Chemical structures of compounds 138–158 reported upon molecular elicitation with global regulatory genes.

a silenced *pga* gene cluster, (accessionno. AY034378) in *Streptomyces* sp. PGA64 resulting in the isolation of two novel anthraquinone aminoglycosides named, gaudimycin D (**160**) and E (**161**). This report validated RGMS as a promising strategy in activating cryptic genes especially after activation of the pga gene cluster which has remained dormant despite more than ten years of continuous efforts using alternative ways.[81]

Ahmed *et al.* applied a reporter-guided screening method to activate cryptic polycyclic tetramate macrolactam gene clusters in *Streptomyces albus* J1074. Herein, the low expressed *S. albus* J1074 PTM gene cluster was awakened using a combination of reporter-guided screening and transposon mutagenesis with *gusA's* selection as a reporter resulting in the production of antimycins (**144–151**) and candicidins (**162**).[82]

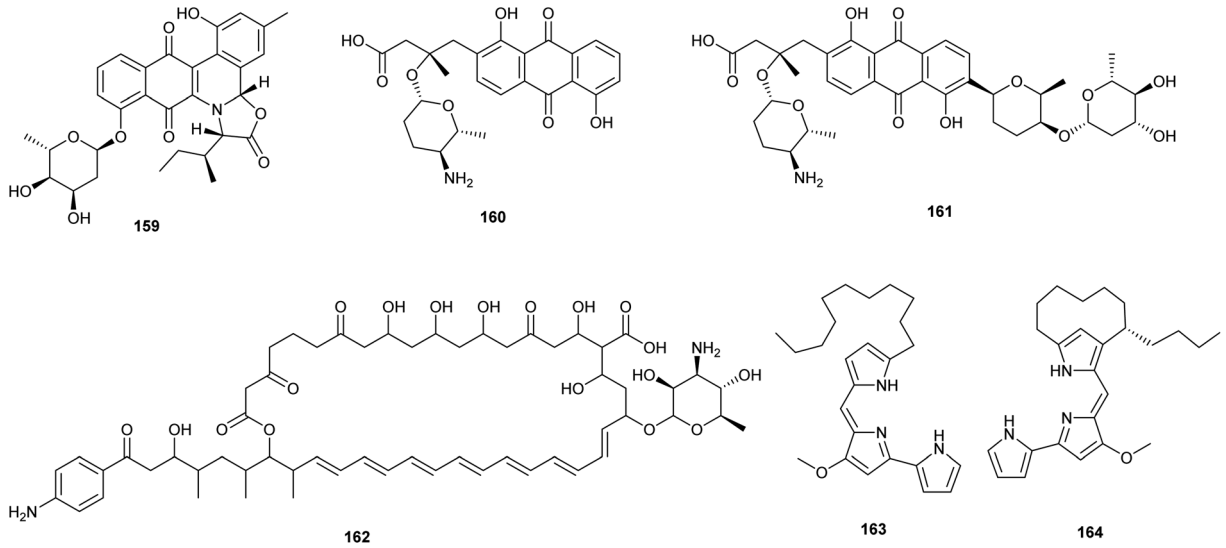

Fig. 9 Chemical structures of compounds **159–164** reported upon molecular elicitation with reporter-guided mutant selection.

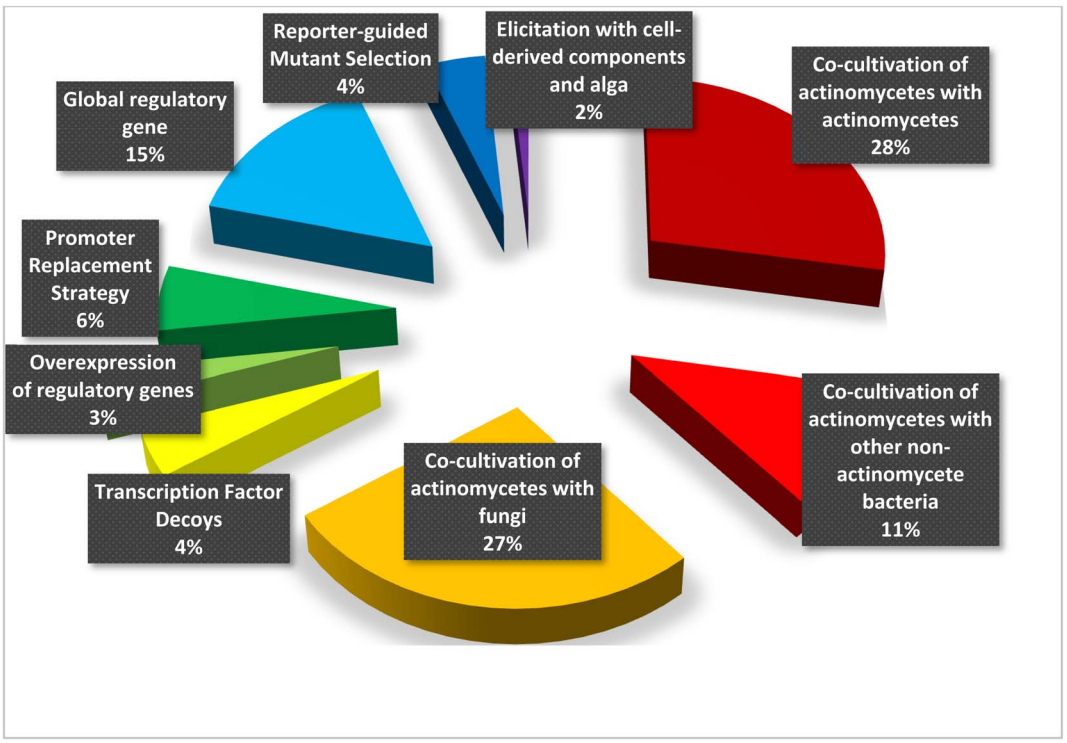

Fig. 10 The total percentage of isolates from the application of different elicitation methods.

6. Conclusions

Recent advances in the whole-genome sequencing of Actinomycetes has uncovered a wide new field of microbial biology and chemistry. Under ordinary growth conditions, most biosynthetic gene clusters found in genomes were found to be "silent". The scientific world has acknowledged that inactive gene clusters contain a significant source of therapeutic compounds. Several techniques for activating silent BGC have been developed. These methods enable the discovery of new metabolite scaffolds and improve the biosynthesis of minor secondary metabolites. The use of cross-species co-cultures, molecular and chemical elicitation for the biosynthesis of novel natural products have emerged as successful techniques in recent years (Fig. 10). In addition, appropriate dereplication methods, such as molecular networking and metabolomics, are needed to analyze the potential uniqueness of the evoked metabolites and prevent isolating existing compounds. Finally,

novel chemical scaffolds from actinomycetes will be found through structural identification of the new hidden products utilizing LC-MS, MS/MS, and NMR.

Author contributions

A. O. H. gathered a complete survey of all isolated compounds; A. O. H. and M. H. A. H. prepared the manuscript; U. R. A. interpreted and revised the results, and wrote the manuscript; S. S. E., R. M. and U. R. A. scientifically discussed the results and contributed to editing of the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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