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A Type I Secreted, Sulfated Peptide Triggers XA21-Mediated Innate Immunity

Sang-Won Lee, *et al.*

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ferentiation. Multiple correspondence analysis of these variables revealed that *H. cydno alithea* differed from other examples of ecological speciation in that divergence is based on a single-locus trait and is not accompanied by postzygotic isolation or background genetic differentiation (fig. S2). Research on other examples of ecological speciation has revealed populations that may also be in an early stage of divergence (*26*–*28*), suggesting that continued examination of these and other systems will reveal a continuum in the trajectory of ecological speciation, as is evident in *Heliconius* butterflies.

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A Type I–Secreted, Sulfated Peptide Triggers XA21-Mediated Innate Immunity

Sang-Won Lee,* Sang-Wook Han,* Malinee Sririyanum, Chang-Jin Park, Young-Su Seo, Pamela C. Ronald†

The rice *Xa21* gene confers immunity to most strains of the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Liquid chromatography–tandem mass spectrometry analysis of biologically active fractions from *Xoo* supernatants led to the identification of a 194–amino acid protein designated Ax21 (activator of XA21-mediated immunity). A sulfated, 17–amino acid synthetic peptide (axY^S22) derived from the N-terminal region of Ax21 is sufficient for activity, whereas peptides lacking tyrosine sulfation are biologically inactive. Using coimmunoprecipitation, we found that XA21 is required for axY^S22 binding and recognition. axY^S22 is 100% conserved in all analyzed *Xanthomonas* species, confirming that Ax21 is a pathogen-associated molecular pattern and that XA21 is a pattern recognition receptor.

In 1995 we showed that the rice *Xa21* resistance gene, which encodes a protein with predicted leucine-rich repeat (LRR), transmembrane, juxtamembrane, and intracellular kinase domains, conferred immunity to diverse strains of the Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (*1*, *2*). Subsequent discoveries in flies (*3*), humans (*4*), mice (*5*), and *Arabidopsis* (*6*, *7*) revealed that animals and other plant species also carry membrane-anchored receptors [Toll in flies; Toll-like receptor 4 (TLR4) in mice and humans] with striking structural similarities to XA21 and that these receptors are also involved in microbial recognition and defense. Like XA21, these receptors typically associate with or carry non-RD (non–Arg-Asp) kinases to control early events of innate immunity signaling (*8*). *Arabidopsis* FLS2 (flagellin-sensitive 2) and EFR (elongation factor receptor) belong to the same class of plant receptor kinases (the LRRXII) as XA21 (*8*, *9*).

Many of these cell surface receptors were later named pattern recognition receptors (PRRs) on the basis of their ability to directly recognize molecules that are conserved across a large class of microbes (*10*, *11*). Such microbial molecules were called pathogen-associated molecular patterns [PAMPs, also known as microbe-associated molecular patterns (MAMPs)] (*12*).

Despite the similarity of the known PRRs to XA21, the classification of XA21 has been debated (*13*, *14*). This is partly because XA21 was discovered before the terms PRR and PAMP were established (*12*) and partly because, under the classical definition of Flor (*15*), XA21 was called a "resistance" gene. Furthermore, because the molecule recognized by XA21 [previously called avrXa21 (avirulence Xa21) and here renamed Ax21 (activator of XA21-mediated immunity)] had not been identified, it was not known whether this molecule was conserved among a large class of microbes—a hallmark of PAMPs (*12*).

We previously identified six *Xoo* genes required for ax21 activity (the *rax* genes), which fall into two functional classes. The first class consists of three genes (*raxA*, *raxB*, and *raxC*) that encode components of a bacterial type I secretion system (TOSS) (*16*). The second class is involved in sulfation, including *raxST*, which encodes a protein with similarity to mammalian tyrosine sulfotransferases (*16*). *Xoo* strains carrying mutations in any of these *rax* genes no longer activate XA21-mediated immunity. None of the identified genes encodes an obvious activator of immunity.

To identify Ax21, we fractionated the supernatant of *Xoo* strain PXO99 cultures on a C18 reversed-phase high-performance liquid chromatography (RP-HPLC) column (Fig. 1A) and carried out bioassays of seven HPLC peptide-enriched fractions (Fig. 1B) using our previously established methods (*17*). An active fraction that was able to trigger XA21-mediated immunity (Fig. 1) was subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) (*18*). Fifteen peptides from the LC-MS/MS spectra matched eight *Xoo* proteins (*18*), including two peptides that corresponded to the N-terminal and C-terminal regions of a 194–amino acid protein encoded by PXO_03968 (boxes in Fig. 1C and fig. S1).

To identify which gene encodes Ax21, we generated *Xoo* strains carrying a mutation in each of the individual genes. Whereas a PXO_03968 knockout strain caused long lesions and grew to high levels on XA21 leaves (Fig. 2), none of the other strains did (fig. S2). These data indicate that the PXO_03968 gene encodes Ax21. We further showed that Ax21 secretion requires *raxA* and *raxC* (fig. S3) (*18*).

Department of Plant Pathology, University of California, Davis, CA 95616, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: pcronald@ucdavis.edu

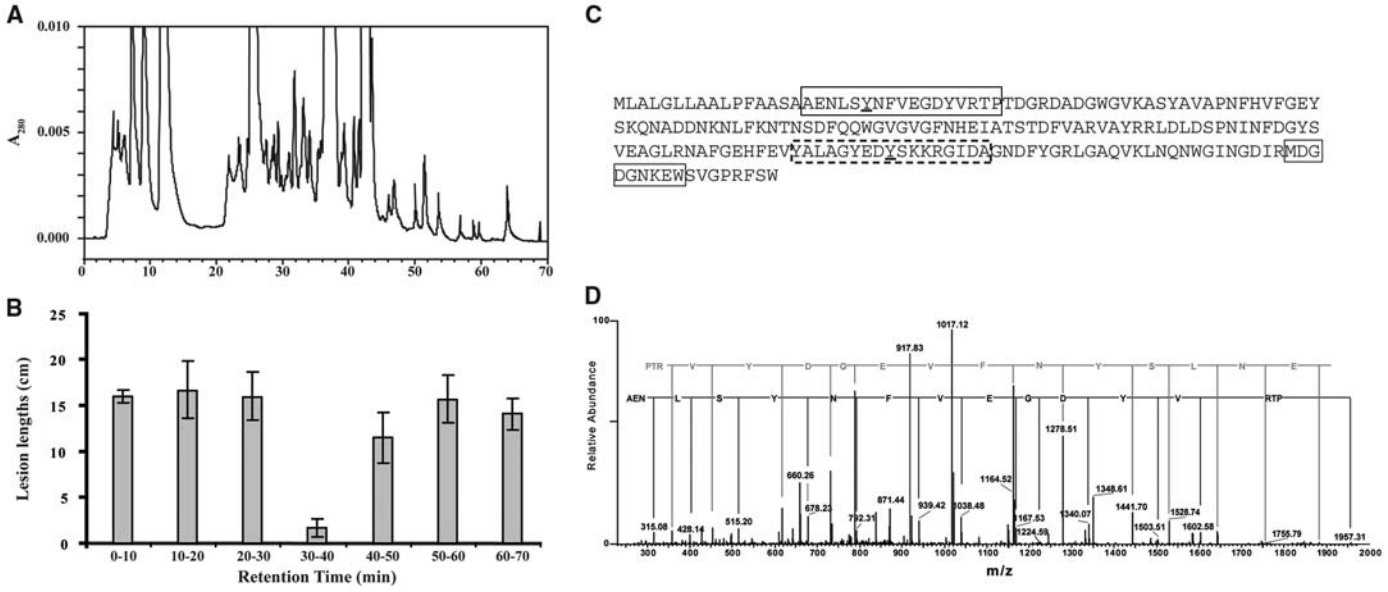

Fig. 1. Isolation of Ax21. (**A**) RP-HPLC elution profile of peptides secreted from *Xoo* strain PXO99 (carrying Ax21 activity). Peptide-enriched samples from the PXO99 supernatant were separated on a reversed-phase C18 column (1 × 250 mm, flow rate 0.05 ml/min) with a 10 to 90% acetonitrile gradient containing 0.1% trifluoroacetic acid. A_{280}, absorbance at 280 nm. (**B**) Lesion length measurements of XA21 rice leaves pretreated with RP-HPLC fractions followed by inoculation with PXO99Δ*raxST*. Lesion lengths were measured 12 days after PXO99Δ*raxST* inoculation. Each value is the mean ± SD from nine inoculated leaves. (**C**) Deduced amino acid sequence of Ax21. The two peptides (boxed) identified from the biologically active fraction were sequenced using LC-MS/MS. Predicted sulfated tyrosines Y22 and Y144 are underlined. The dashed box indicates one of the peptide used in the Ax21 bioassay shown in Fig. 3. (**D**) Mass (LTQ) spectrum of the axY22 peptide corresponding to the N-terminal region [first box in (C)] of Ax21. The spectrum corresponding to the peptide derived from the C-terminal region [second box in (C)] of Ax21 is shown in fig. S1.

To test the importance of the putative tyrosine sulfation sites on Ax21 (*19*), we synthesized seven peptides: two carrying sulfated tyrosines in the target residues [Tyr^{22} and Tyr^{144} (Y22 and Y144)], two carrying nonsulfated tyrosines, two carrying alanines in place of the tyrosines, and one corresponding to the C-terminal region of Ax21 (Fig. 3A) (*19*). XA21 rice leaves were pretreated with each peptide (100 μM in water). The 17–amino acid peptide carrying Y22 sulfation (axY^S22) activated XA21-mediated immunity (Fig. 3B). To further quantify this response, we characterized the activity of the axY^S22 synthetic peptide by growth curve analysis. Pretreatment of XA21 rice leaves with the axY^S22 peptide triggered resistance to PXO99Δ*raxST*, as reflected in a reduction in PXO99Δ*raxST* population growth by three orders of magnitude. The nonsulfated peptide (axY22) was unable to trigger XA21-mediated immunity (Fig. 3C).

Bioassays with 17 axY^S22 peptide variants carrying alanine substitutions identified eight amino acids critical for XA21-mediated immunity (fig. S4A) (*18*). A concentration of 1 μM is sufficient for PAMP activity (fig. S4B) (*18*).

In coimmunoprecipitation experiments with hemagglutinin (HA)–tagged axY^S22 and extracts from leaves carrying a Myc-tagged XA21 protein (*18*), we observed labeling of a band migrating at 140 kD by SDS–polyacrylamide gel electrophoresis (PAGE) with antibodies to both Myc and HA (Fig. 4). The presence of 5- to 10-fold excess untagged axY^S22 peptide suppressed the labeling of this band, whereas $flg22_{ave}$ from

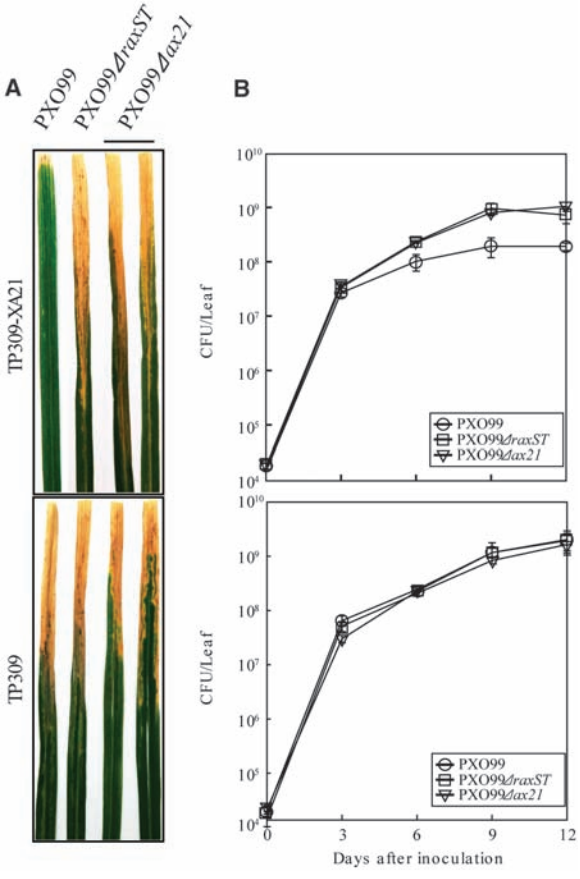

Fig. 2. A mutation in *ax21* abolishes Ax21 activity. (**A**) Lesion lengths of rice leaves measured 12 days after inoculation with *Xoo* strains PXO99, PXO99Δ*raxST*, or PXO99Δ*ax21*. Suspensions of each strain [10^8 colony-forming units (CFU)/ml] were scissor-inoculated onto rice leaves (TP309-XA21, resistant to PXO99; TP309, susceptible to PXO99). Images are representative of five independent experiments. (**B**) Growth of PXO99, PXO99Δ*raxST*, and PXO99Δ1*ax21* populations in inoculated rice leaves. Bacteria were extracted from the leaves at 0, 3, 6, 9, and 12 days after inoculation, plated on selective media after serial dilution, and colonies counted after a 3-day incubation at 28°C. Each value is the mean ± SD from nine inoculated leaves.

the rice pathogen *Acidovorax avenae* had no effect on axY^{S}22-XA21 binding (Fig. 4). These experiments demonstrate that XA21 is required for axY^{S}22 binding and recognition.

Sequence analysis indicates that Ax21 is highly conserved in *Xoo* strains (KACC 10331 and MAFF 311018, both 98% identity), *X. campestris* pv. *campestris* (90%), *X. axonopodis* pv. *glycinea* (92%), *X. axonopodis* pv. *vesicatoria* 85-10 (*Xav*) (92%), and *X. oryzae* pv. *oryzicola* (98%) (fig. S5). The 17–amino acid axY^{S}22 sequence is 100% conserved in these strains. *Xylella fastidiosa* and the opportunistic human pathogen *Stenotrophomonas maltophilia* also carry putative Ax21 orthologs (48% and 61%, respectively) and show 77% and 65% similarity, respectively, to the axY^{S}22 sequence (fig. S5).

Because *Xav* carries predicted orthologs for Ax21, *raxST*, *raxA*, and *raxB*, which we have previously shown to be required for Ax21 activity (*16*), we hypothesized that *Xav* would express Ax21 activity. Indeed, we found that pretreatment of XA21 rice leaves with supernatants from wild-type *Xav*, but not *Xav* strains carrying a deletion of *ax21* (*18*), can activate XA21-mediated immunity (fig. S6). These results indicate that the *ax21* ortholog in *Xav* possesses the predicted biological activity.

One of the key aspects of the definition of PAMPs is that they "are conserved within a class of microbes" (*10*). As a result of the explosion of studies on PRRs and PAMPs in both plant and animal systems, it has now become clear that PAMPs can be conserved quite widely across genera (e.g., flagellin) or more narrowly within a genus (e.g., Pep13) (*20*) and, further, that sequence variation and posttranslational modifications can modulate PRR-dependent pathogen recognition (*18*, *21*).

In the XA21-Ax21 system, the axY^{S}22 peptide sequence is invariant in all sequenced *Xanthomonas* species. Sulfation provides specificity to the system, just as flagellin or lipopolysaccharide recognition in some hosts is modulated by glycosylation or acylation, respectively (*21*, *22*). Thus, Ax21 is a PAMP that satisfies the genetic definition of an avirulence factor because the presence or absence of sulfation on the conserved 17–amino acid epitope is decisive for its ability to trigger XA21-mediated immunity. Similarly, *Xa21* is a disease resistance gene because it is the single polymorphic determinant in rice that confers resistance to strains of bacteria expressing sulfated Ax21, and it is also a PRR because it is required for recognition of a particular modified peptide epitope that is conserved across a microbial genus.

Thus, our data provide another example of bacteria-host interactions that can be attributed to the presence of genes encoding proteins (e.g., sulfotransferases, glycosylases, and acetylases) that modify conserved peptide epitopes (*21*–*25*). Such examples indicate that successful pathogens of plants and animals have evolved methods of altering the PAMP to avoid detection by the host PRR. Conversely, the presence or absence of a particular PRR can have a marked effect on the resistance of the host to infection. Just as plants deficient in XA21 or FLS2 exhibit reduced resistance to phytopathogens, mice deficient for TLR4 or TLR2 are altered in their response to *Mycobacterium tuberculosis* infection (*22*). These studies have led to a convergence in our understanding of the molecular mechanisms governing the specificity of host-microbe interactions in plants and animals.

Our results thus demonstrate that the definitions of PAMPs and *avr* genes and those of disease resistance genes and PRRs cannot be strictly separated. Future usage of these terms should reflect the concepts presented by Medzhitov (*12*) and Flor (*15*), and must also take into account subsequent discoveries of the effects of PAMP polymorphism and posttranslational modifications. In plants, a disease resistance gene is merely an allele in the host genotype that confers resistance in a particular interaction. Such genes can encode diverse proteins (although, to date, the great majority encode intracellular nucleotide-binding LRR

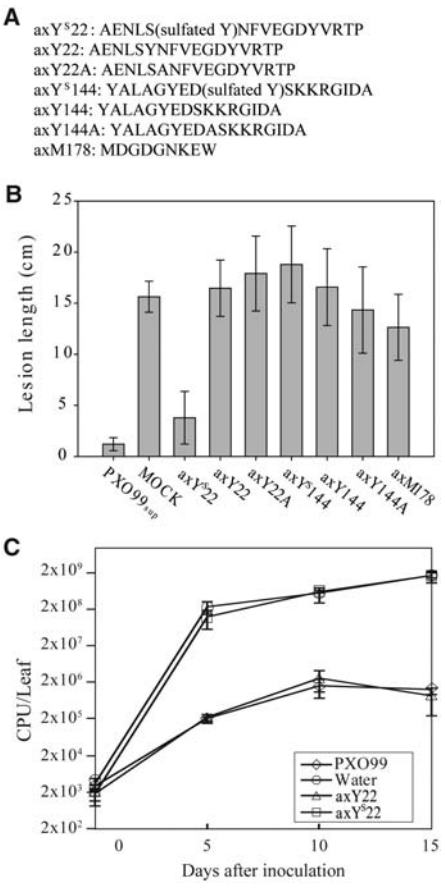

Fig. 3. The axY^{S}22 peptide is sufficient to trigger XA21-mediated immunity. (**A**) Synthetic peptides, including three corresponding to the N-terminal region of AX21 (axY^{S}22, axY22, and axY22A), three corresponding to the central region (axY^{S}144, axY144, and axY144A), and one corresponding to the C-terminal region (axM178), were tested for activity. Abbreviations for amino acid residues: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (**B**) Five hours after peptide pretreatment, leaves were inoculated with PXO99Δ*raxST* and the lesions measured 12 days later. Each value is the mean ± SD from six leaves. (**C**) Growth of PXO99Δ*raxST* populations over time. TP309-XA21 leaves were pretreated with PXO99 supernatant (PXO99$_{sup}$), water, or the synthetic peptides (axY^{S}22 and axY22, 100 μM each). Bacterial cells were extracted from the leaves at 0, 5, 10, and 15 days after inoculation, plated on selective media after serial dilution, and colonies counted after a 3-day incubation at 28°C. Each value is the mean ± SD from eight inoculated leaves.

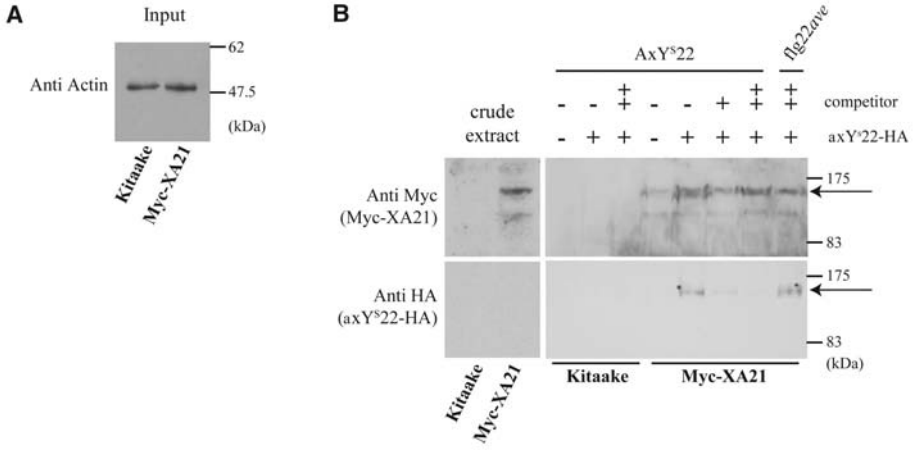

Fig. 4. XA21 is required for axY^{S}22 binding. HA-tagged axY^{S}22 cross-links to a 140-kD polypeptide that is immunoprecipitated by an antibody to Myc (Myc-XA21). (**A**) Before immunoprecipitation, the loading of equal amounts of protein (50 μg) from Kitaake and Myc-XA21 leaf extracts was confirmed using an antibody to actin (input). (**B**) Leaf extracts were incubated with 1 mM HA-axY^{S}22 in the presence (+, 5 mM; ++, 10 mM) or absence (–) of the competitors axY^{S}22 lacking the HA tag or flg22$_{ave}$. After binding, cross-linking was initiated by the addition of sulfo-Ethylene Glycol bis (Succinimidyl Succinate). Duplicate protein gels were analyzed after separation by SDS-PAGE using antibodies to Myc (top) and to HA (bottom). Myc-XA21 and a proteolytic cleavage product of Myc-XA21 were detected at 140 and 110 kD, respectively, as reported previously (*32*). Arrows indicate the XA21 and Ax21-XA21 complexes.

proteins) (*2*, *26*–*28*). Hence, the term "*R* gene" is merely operational and mechanistically agnostic.

Likewise, the term "avirulence gene" remains useful as a broad term that indicates a gene that encodes any determinant of the specificity of the interaction with the host. Thus, this term can encompass some PAMPs and pathogen effectors (e.g., bacterial type III effectors and oomycete effectors) as well as any genes that control variation in the activity of those molecules (*29*–*31*)].

In the future, a diverse array of PAMPs from plant pathogens will likely be discovered. Many of these will almost certainly serve as ligands for the large class of predicted orphan PRRs present in the genomes of plant species (371 in rice; 47 in *Arabidopsis*) (*8*).

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Small-Molecule Activators of a Proenzyme

Dennis W. Wolan, Julie A. Zorn, Daniel C. Gray, James A. Wells*

Virtually all of the 560 human proteases are stored as inactive proenyzmes and are strictly regulated. We report the identification and characterization of the first small molecules that directly activate proenzymes, the apoptotic procaspases-3 and -6. It is surprising that these compounds induce autoproteolytic activation by stabilizing a conformation that is both more active and more susceptible to intermolecular proteolysis. These procaspase activators bypass the normal upstream proapoptotic signaling cascades and induce rapid apoptosis in a variety of cell lines. Systematic biochemical and biophysical analyses identified a cluster of mutations in procaspase-3 that resist small-molecule activation both in vitro and in cells. Compounds that induce gain of function are rare, and the activators reported here will enable direct control of the executioner caspases in apoptosis and in cellular differentiation. More generally, these studies presage the discovery of other proenzyme activators to explore fundamental processes of proenzyme activation and their fate-determining roles in biology.

Activation of proteases triggers a myriad of biological events, such as apoptosis and blood clotting, both inside and outside of the cell (*1*). Proteases are generally stored as inactive proenzymes that are usually activated by upstream proteases or by themselves. These activation events may sometimes involve binding a protein partner or, in rare instances, interaction with a natural small molecule (*2*) or peptide (*3*). In the case of autoproteolysis, the proenzyme must achieve not only an active state, but also one in which the sites of proteolysis are exposed. In situ activation of specific proproteases with synthetic small molecules could uncover new molecular principles in zymogen activation and would facilitate direct control of these important processes in biology.

The executioner procaspases, consisting of procaspases-3, -6 and -7, represent excellent initial candidates for discovery of small-molecule protease activators. Caspases are a family of homodimeric cysteine proteases responsible for many of the fate-determining processes in cell biology, including apoptosis, innate immune signaling, early stages of stem cell differentiation, and cellular remodeling (*4*–*6*). As with most proteases, caspases are synthesized as inactive procaspases, or zymogens, and are activated by upstream proteolysis or autoproteolysis. Previous studies have shown that the mature active caspases are intrinsically dynamic (*7*, *8*) and sample both an "on state" and an "off state" that structurally resemble the zymogen-like conformation (*9*, *10*). Small molecules have been found to trap these two forms of the mature enzyme (*11*, *12*). We reasoned that if the procaspases existed in a similar dynamic equilibrium of off and on states, it might be possible to find small molecules that promote autoproteolytic activation via stabilization of an on-state conformation. Executioner procaspases are particularly good targets as they are susceptible to rapid activation by both upstream proteases and self-proteolysis. Thus, any activation would be accentuated in trans by autocatalytic activation. Moreover, these particular caspases are essential for executing the final processes of apoptosis, and specific activation by a small molecule would elicit robust and precise phenotypic responses within cells.

High-throughput screening was employed to identify compounds that could promote autoproteolytic activation of procaspase-3 at physiological concentrations [for HTS design, see (*13*)]. A dozen compounds out of 62,000 promoted >20-fold activation of procaspase-3 (fig. S1) and were resynthesized to validate their chemical composition. To warrant further analysis, dynamic light-scattering was used to select compounds with solubilities greater than 100 μM, and a β-lactamase inhibition assay was performed to discard promiscuous aggregators (*14*). The most robust procaspase activator fulfilling these criteria was compound 1541, a substituted phenylimidazopyridine-methoxy coumarin with a median effective concentration (EC_{50}) for activation of 2.4 μM (Fig. 1A). Mass spectrometry revealed that the compound did not covalently label either the mature or proenzyme forms of caspase-3, nor was 1541 modified by enzyme hydrolysis.

Departments of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology, University of California, San Francisco, Byers Hall, 1700 4th Street, San Francisco, CA 94158, USA.

*To whom correspondence should be addressed. E-mail: jim.wells@ucsf.edu