Study of amino acid-derived 3-acyltetramic acids as herbicidal agents

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Abstract

The growing problem of herbicide resistance necessitates the development of novel herbicidal active ingredients, together with other integrated weed management approaches. Natural products are a major source of inspiration for novel actives. In previous research, we identified a 3-acyltetramic acid of microbial origin that inhibited algal growth in marine biofilms at least in part through inhibition of photosystem II. In this work, we demonstrate the herbicidal effect of this lead compound and construct multiple libraries to test the impact of the different substituents of the central scaffold in order to study the structure-activity

relationships. Amongst these analogues, the highest activities were found for medium to long chain acyl groups and apolar secondary amino acid residues. Finally, we provide first insights into the herbicidal mechanisms and present preliminary field-trial and ecotoxicological results for TA12-Pro, the most active analog in our library. Together, this research shows the potential of 3-acyltetramic acids for herbicide development.

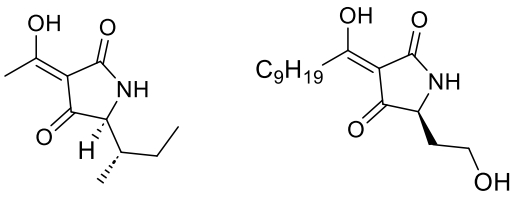
Keywords:

herbicides, agrochemistry, tetramic acids, chemical synthesis

Introduction

Chemical herbicides continue to be a key pillar of integrated weed control programs, but require careful resistance management to preserve their efficacy.[1] However, resistance management is increasingly complicated by overreliance on a limited number of active substances due to the simultaneous phasing out of existing actives on health and environmental grounds and a lack of new actives coming to market to replace them.[1]

Natural products are a crucial source of novel lead compounds for crop protection chemists, including for herbicide development.[2] Tetramic acids are a large class of natural products produced predominantly by fungi and bacteria that have received intense interest from medicinal and agrochemical researchers owing to their wide range of bioactivities (including, but not limited to, antitumor, antibacterial, insecticidal, phytotoxic and amoebicidal activities).[3–7] Of particular relevance to herbicide researchers is the observation that several micro-organisms produce 3-acyltetramic acids (3-ATAs) which act as potent inhibitors of photosystem II. Two notable examples are the mycotoxin tenuazonic acid (TeA, **1**), produced by a wide range of plant-pathogenic fungi including *Alternaria* spp., *Pyricularia oryzae* and *Aspergillus* spp.[8], and 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione (TA12, **2**), produced by various marine bacteria shown to inhibit algal growth (**Figure 1**).[9]



1 (TeA) **2** (TA12)

Figure 1: *Structures of tenuazonic acid (TeA, **1**) and TA12 (**2**).*

Significant research effort has been spent on trying to turn compound **1** into a broad-spectrum post-emergence herbicide, as discussed by Chen and Qiang.[8] However, TeA **1** is a mycotoxin with an animal- and human health risk at the herbicidal concentrations, which makes it unlikely to find acceptance.[10]

Moreover, the efficacy of compound **1** as a PSII inhibitor appears more modest than that of TA12 (**2**): its half-inhibitory concentration in algae appears to be more than an order of magnitude higher than that of compound **2**.[9] For these reasons, we hypothesized that compound **2** might be a more promising lead candidate for herbicide development than compound **1**. Furthermore, the limited body of literature published on the biological activity of compound **2** and other medium- and long-chain 3-ATAs suggests they might have an important role in microbial ecology in processes as diverse as bacterial-algal competition in marine biofilms[9] and bacterial defense against amoebal predation.[6,7] Further insights into the biological activity and efficient synthesis of long-chain 3-ATAs might thus prove useful for the wider field of chemical ecology.

In this study, we build on the preliminary results obtained in algae with compound **2** and explore the potential of 3-ATAs as scaffolds for novel herbicidal compounds. We present a readily accessible synthetic methodology for 3-ATAs and use it to generate a novel library of 3-ATAs. The effect of the amino acid moiety and length of the side chain on the phytotoxicity of these compounds is assessed systematically via high-throughput multispectral imaging-based assays on leaf disks and whole seedlings. Moreover, we provide first insights into the herbicidal mechanisms of 3-ATAs. Finally, a preliminary ecotoxicological assessment and field evaluation of the most active analog were conducted to gain further insights into the potential of 3-ATA as leads for herbicide development.

Materials and methods

Full details on the equipment, used reagents, plant experiments, synthesis of the starting products and characterisation data of the final compounds can be found in the supporting information.

Synthesis of TA12 (2)

TA12 ***(2)*** ((S,Z)-3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione) was synthesized according to the procedure described by Stock *et al*.[9]

General procedure for the synthesis of tetramic acids 5-36

The synthesis of the *N*-acylated amino acids **4** was adapted from Klapper *et al*.[7] The corresponding oxo ester **3** (6 mmol, 1 equiv.), DMAP (18 mmol, 3 equiv.) and the corresponding L-amino acid methyl ester hydrochloride (7.2 mmol, 1.2 equiv.) were dissolved in 45 mL toluene and this mixture was stirred for 24 hours under reflux. To quench the reaction mixture, an equal volume of 1.2 mol L^{-1} HCl was added. The resulting phase was extracted three times with 50 mL EtOAc, the organic phases were combined and dried over $MgSO_4$ and the solvent was removed *in vacuo*. These crude compounds **4** were all obtained as dark oils and were used in subsequent reactions without further purification.

The synthesis of the tetramic acids ***5-36*** was adapted from Stock *et al*.[9] The corresponding *N*-(3-oxoalkanoyl)-L-amino acid methyl ester **4** (6 mmol, 1 equiv.) was dissolved in 10 mL dry methanol under an argon atmosphere in a 50 mL dried flask. Subsequently, 14.4 mL of a 0.5 mol L^{-1} solution of sodium methoxide (7.2 mmol, 1.2 equiv.) in dry methanol was added and this mixture was stirred for 3 hours under reflux conditions. After reaction, the mixture was poured into 10 mL of water and extracted with 10 mL of diethyl ether. This organic phase was discarded and the aqueous phase was acidified with 2 mol L^{-1} HCl until a pH of 2 was reached. This aqueous phase was then extracted three times with 10 mL of diethyl ether. The combined organic phases were dried over $MgSO_4$ and the solvent was removed *in vacuo*. The final residue was then purified by automatic flash chromatography on reversed-phase silica gel (solvent A: water, solvent B: acetonitrile, gradient: 30% B to 100% B).

Chemical treatments

All compounds used in this study were formulated with DMSO as a co-solvent and Tween 20 as a surfactant. A concentrate was produced that consisted of 60% active product, 35% DMSO and 5% Tween 20 (by mass), which was heated at 60 °C for 10 minutes with vortex mixing to create a stable emulsion.[11] This concentrate was diluted with distilled water until the target concentration and vortexed vigorously immediately prior to use. For leaf-disk assays, this dilute suspension was applied by adding two 5 µL droplets on a leaf disk with a micropipette, whereas for spray assays with seedlings the solution was added to an atomizer and seedlings were sprayed until run-off.[12] In all experiments, the blank formulation (containing only DMSO, water and Tween 20) was included as a negative control, and the photosystem II-inhibiting herbicide diuron (DCMU) was included as a positive control at a concentration of 10 mmol L^{-1}. Full plant materials and experimental setups can be found in the supporting information.

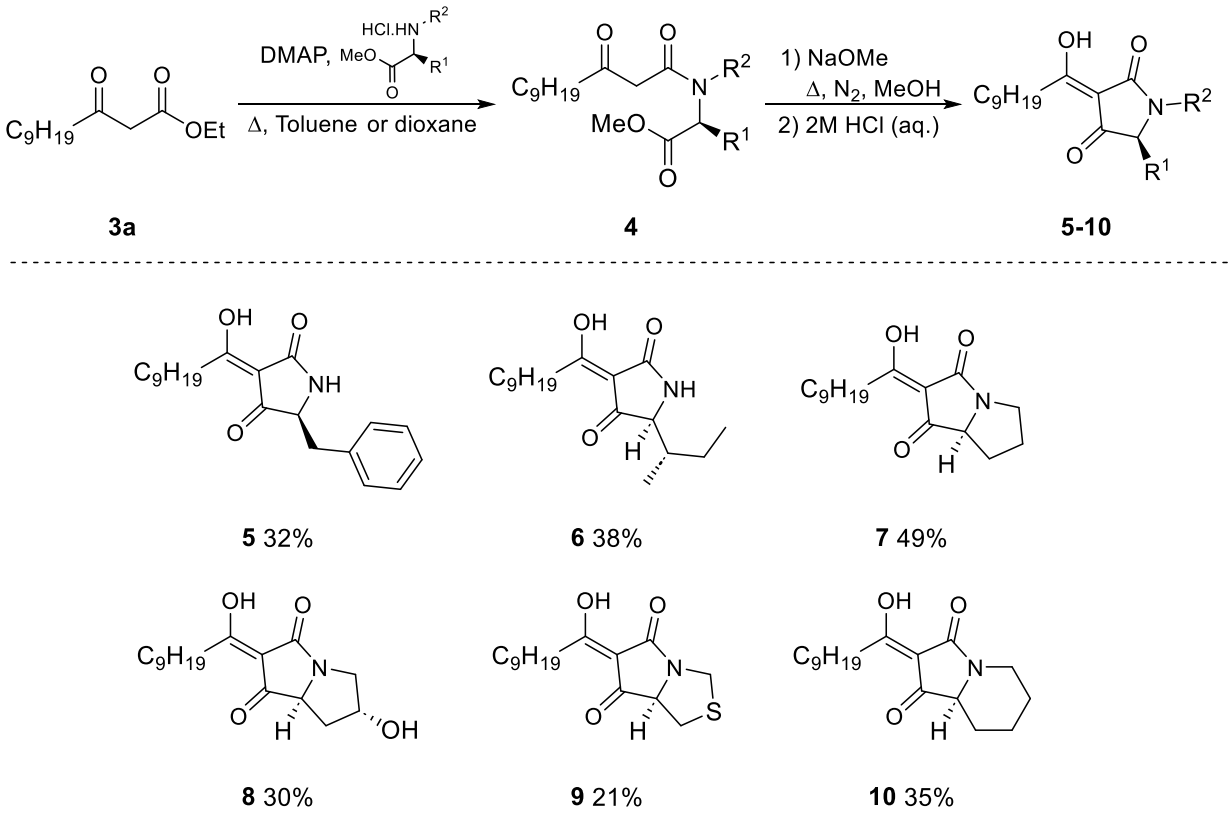
Assessment of toxicity using the PathoViewer system

F_v/F_m of leaf disks or seedlings was measured using the PathoViewer multispectral imaging system described in previous work,[13,14] and images were processed using the CropReporter software (v. 5.4.6-64b, PhenoVation). Herbicidal activity was assessed by measuring F_v/F_m, the maximum quantum efficiency of photosystem II (PSII).[15] This parameter is a reliable quantitative proxy for stress in leaf tissues due to its robust correlation to the severity of biotic and abiotic stresses,[15] including herbicide damage.[16] F_v/F_m was measured after a fifteen-minute dark adaptation period. In between treatments, leaf disks or seedlings were incubated in a growth chamber (21 °C, 14/10 hours light/dark, 120 µmol m^{-2} s^{-1} at canopy level). F_v/F_m values were then analyzed by ANOVA followed by Tukey's Honest Significant Difference Test for pairwise comparisons; homoscedasticity and normality assumptions were verified using diagnostic plots. For binary variables (mortality), Fisher's exact test was used. All statistical analyses were conducted in R (v. 4.3.0).

Results and discussion

3-Acyltetramic acids show non-systemic phytotoxicity in an in vitro bioassay

To assess the potential of 3-ATAs as herbicidal agents, our starting point is the homoserine lactone-derived TA12 (**2**), the target structure of our previous research in algae.[9] The well-established synthetic methodology described there for the synthesis of *N*-acyl homoserine lactones and their derived tetramic acids[17–19] was adapted in this study to ensure compatibility with a larger set of target products. Following previous efforts by Stallforth and coworkers,[7] we started our synthetic methodology from the corresponding ethyl β-ketoesters **3**, which yield the desired β-keto amides **4** after heating under reflux in the presence of amino acid esters and 4-(dimethylamino)pyridine (DMAP). Subsequent Lacey-Dieckmann rearrangement with sodium methoxide in methanol then yields the target tetramic acids (**5-10**).[9,17,20] As an initial screening, we synthesized six TA12-analogues with different amino acid (AA) moieties: the primary AAs L-phenylalanine (**5**) and L-isoleucine (**6**) and the secondary AAs L-proline (**7**), L-hydroxyproline (**8**), L-cysteine-derived thiaproline (**9**) and L-pipecolic acid (**10**). These proposed analogues could all be synthesized swiftly in moderate to good yields via this two-step process (Scheme 1, full details in the Supplementary information). Of these six analogues, only the L-phenylalanine[21] and L-proline[6] analogues were described previously.



*Scheme 1: Two-step synthesis for analogues **5-10** starting from ethyl 3-oxododecanoate **3a**.*

These compounds **5-10** were evaluated for phytotoxicity by applying two 5 µL droplets of a 10 mmol L^{-1} compound solution to tomato leaf disks in 24-well plates and monitoring changes in F_v/F_m (**Figure 2**). A concentration of 10 mmol L^{-1} was chosen as a reference concentration for further analysis because it was the lowest concentration that reliably resulted in complete loss of photosynthesis at application sites in the leaf disk assay for at least some of the tested compounds. F_v/F_m is a reliable measure of photosynthetic efficiency and thus indirectly of the health and integrity of leaf tissues with proven utility in screening herbicidal compounds.[15,16] Out of the seven tested AA moieties, only one compound (**5**) did not significantly reduce F_v/F_m at the droplet application site. The other six showed considerable variation in the magnitude of their effect on F_v/F_m, with only the L-proline analog TA12-Pro **7** showing sufficiently high activity to significantly reduce the F_v/F_m of the leaf disk as a whole (-32%, p = .0019). The effect of the 3-ATAs stands in contrast to the positive control treatment, the PSII-inhibiting herbicide diuron (DCMU), which achieved a uniform reduction in F_v/F_m throughout the leaf disk (**Figure 2**). Our data also confirms that the formulation

used for the 3-ATAs and DCMU is itself non-phytotoxic, as no changes in F_v/F_m were observed compared to a water control (+1%, p = 1.00).

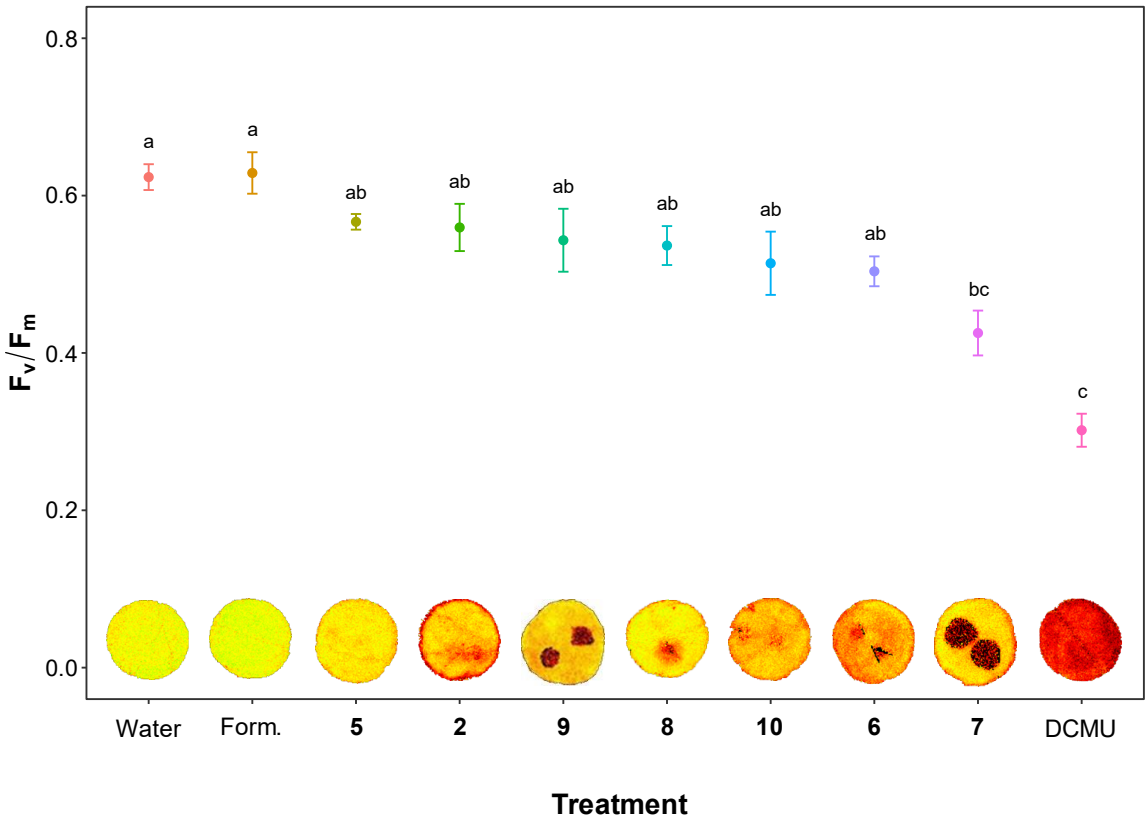
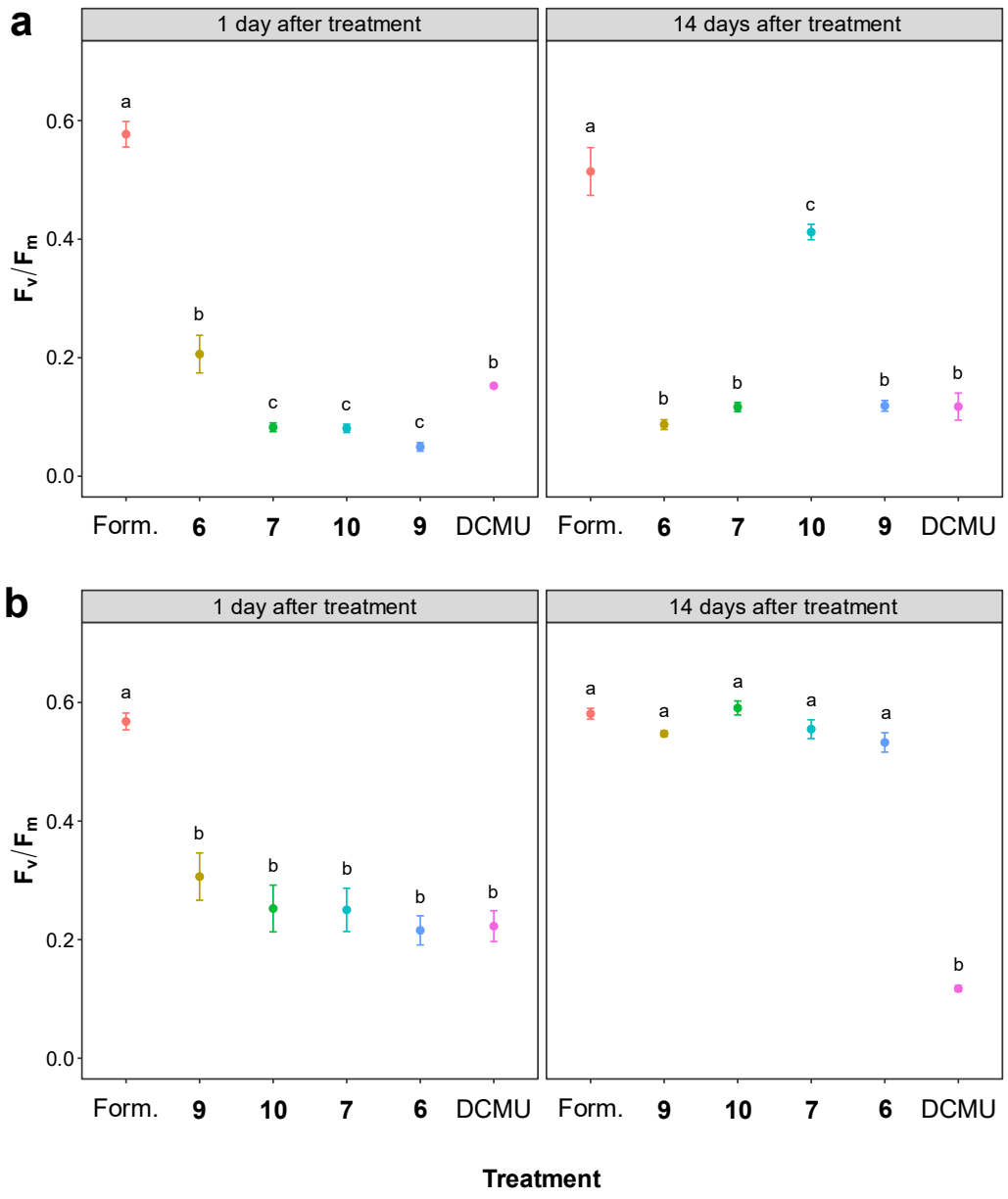


Figure 2: *Effect of 3-ATAs* ***2*** *and* ***5-10*** *on F_v/F_m in tomato leaf disks 96 hours after application. Two 5 µL droplets of a 10 mmol L^{-1} compound solution were applied to each 1.1 cm diameter tomato (*Solanum lycopersicum *'Moneymaker') leaf disk floating in distilled water. A representative F_v/F_m image of a leaf disk is shown for each treatment (color scale: yellow corresponds to highest F_v/F_m, values, orange to intermediate values and red-black indicate to low or very low values). The abbreviation 'Form.' is used to indicate formulation control (i.e. without active ingredient). Error bars indicate mean ± SEM, N = 4. Treatments with different letters differ significantly according to Tukey's Honest Significant Difference test (p < .05).*

The in vitro phytotoxicity of 3-ATAs translates to herbicidal activity against agronomically important weed species

To validate the herbicidal effect of the 3-ATAs in intact seedlings of an agronomically relevant weed, the four 3-ATAs that showed the greatest activity in the leaf disk assay (compounds **6**, **9**, **10** and TA12-Pro **7**) were sprayed on seedlings of the broadleaf weed *Amaranthus retroflexus* L. at a concentration of 10 mmol L^{-1} (**Figure 3a**). Twenty-four hours after application, seedlings treated with each of the four 3-ATAs showed an F_v/F_m value similar to or lower than that of DCMU-treated seedlings, indicating complete death of treated tissues. To assess possible regrowth, seedlings were monitored for a further fourteen days. Seedlings treated with the pipecolic acid derivative **10** showed regrowth, whereas those treated with DCMU or the three other tested 3-ATAs did not.



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174 ***Figure 3:*** *Effect of 3-ATAs* ***6-7*** *and* ***9-10*** *on* F_v/F_m *of intact seedlings of* Amaranthus retroflexus ***(a)*** *and* Echinochloa crus-galli ***(b)***
175 *one and fourteen days after treatment. Seedlings were sprayed until run-off with a 10 mmol* L^{-1} *compound solution using an*
176 *atomizer. The abbreviation 'Form.' is used to indicate formulation control (i.e. without active ingredient). Error bars indicate*
177 *mean ± SEM, N = 4. Treatments with different letters differ significantly according to Tukey's Honest Significant Difference test*
178 *(p < .05).*

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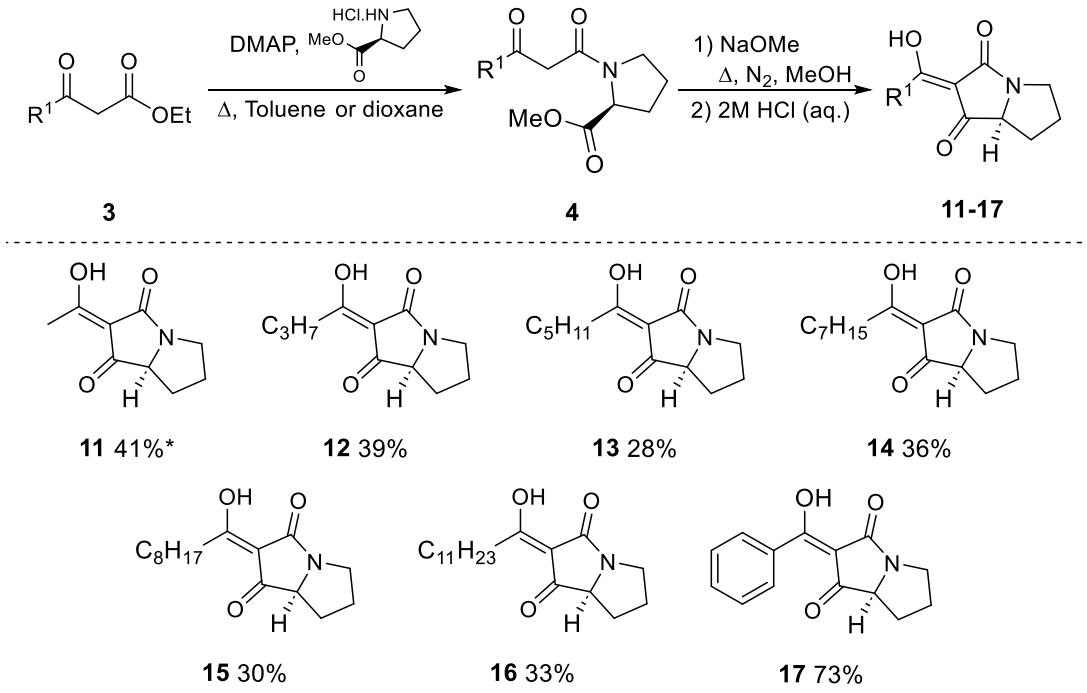
The same assay was conducted with the monocot weed *Echinochloa crus-galli* L. P. Beauv. (**Figure 3b**). All four 3-ATAs achieved the same reduction in F_v/F_m as DCMU one day after treatment, but fourteen days after treatment all 3-ATA-treated seedlings showed regrowth whereas DCMU-treated seedlings did not. This observation is consistent with a non-systemic contact effect, as the meristem of grasses is located close to the ground in a protective sheath and is thus hard to destroy with contact products. Increasing compound concentration from 10 mmol L^{-1} to 50 mmol L^{-1} and even 100 mmol L^{-1} did not prevent regrowth in *E. crus-galli* (data not shown). Together, these results confirm the contact herbicide activity of 3-ATAs suggested by our *in vitro* assay.

Intrigued by these results, we wanted to continue our investigation of this scaffold and evaluate the importance of the acyl side chain (position 3 in the pyrrolidine ring) and the AA residue (positions 1 and 5) in this scaffold. Subsequently, we investigated the importance of the exocyclic enolic proton and the light dependence of our activity, in order to have a more complete view on the activity of our products. Finally, we address the practical application of our products with a field trial and a preliminary ecotoxicological assessment.

The acyl side chain significantly affects the herbicidal activity of 3-ATAs

To evaluate the role of side chain length in herbicidal activity, the most active AA moiety (L-proline, **7**) was selected and analogs with acyl side chain lengths varying from two (**11**) to twelve (**16**) carbon atoms were evaluated for herbicidal activity. In addition, an analog with an odd chain length (**15**) and a benzoyl group instead of an acyl group (**17**) were also included. With the exception of the compound with the shortest chain length, **11**, these compounds could all be synthesized in moderate to good yields via the methodology described in Scheme 1 (see compounds **12-17**, Scheme 2). The only exception was compound **11**, for which 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one was used instead of ethyl acetoacetate, yielding the

target product in a 48% yield over two steps. Most of these analogues have been described previously in the work of Stallforth and colleagues, with the exception of compounds **15**, **16** and **17**.[6]



*Scheme 2: Synthesis of acyl chain analogues **11-17** of TA12-Pro **7**. *2,2,6-trimethyl-4*H*-1,3-dioxin-4-one was used as starting product instead of ethyl acetoacetate.*

Our data show a clear increase in herbicidal efficacy with increasing acyl chain length against an additional agronomically important monocot weed *Alopecurus myosuroides* Huds. (**Figure 4)**: treatment with 10 mmol L^{-1} of compounds **11** and **12** does not significantly reduce F_v/F_m (-2% and –18% respectively, p = 1.00 and p = .28), whereas side chains of six carbon atoms (**13**) or more achieve reductions comparable to or greater than DCMU at the same concentration. Compound **15**, the only analog with an odd side chain length, shows a statistically identical performance to compounds **14** and **7** (-56% for **15** *vs*. –61% and –62% for **14** and TA12-Pro **7**), which shows that there is no clear difference between odd- and even-numbered side chains. On the other hand, replacement of the acyl side chain with a benzoyl group leads to complete loss of activity (-3% for compound **17** *vs*. formulation control, p = 1.00). Importantly, while several 3-ATAs could kill directly affected foliage at 10 mmol L^{-1}, all treated seedlings showed regrowth

within one to two weeks even at 50 mmol L^{-1} (data not shown). This further confirms that the 3-ATAs show only contact activity, with no evidence for systemic translocation.

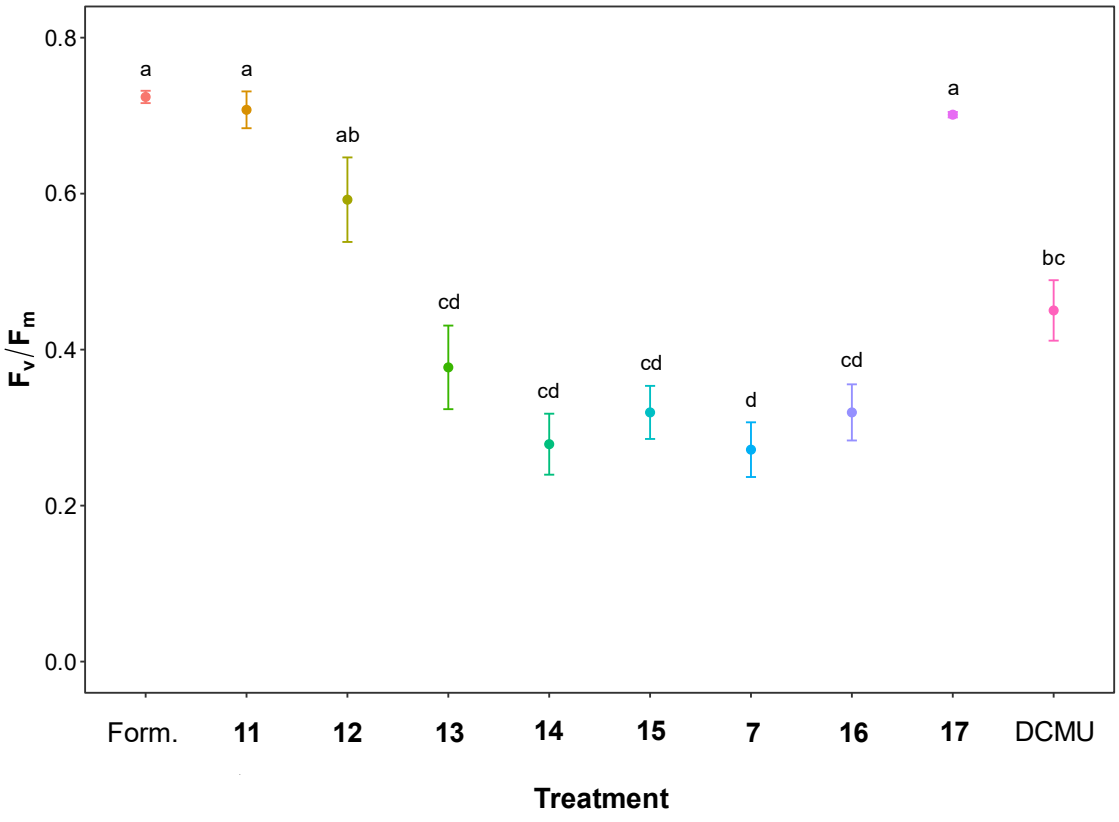


Figure 4: *Effect of L-proline-derived tetramic acids with varying side chains on seedlings of* Alopecurus myosuroides. *F_v/F_m was measured 72 hours after spraying until run-off with a 10 mmol L^{-1} solution. The abbreviation 'Form.' is used to indicate formulation control (i.e. without active ingredient). Error bars indicate mean ± SEM, N = 4. Treatments with different letters differ significantly according to Tukey's Honest Significant Difference test ($p < .05$).*

To further explore the role of the side chain length, spray experiments were also conducted in two broadleaf weeds, *Amaranthus retroflexus* L. and *Stellaria media* L. Vill. (**Figure 5**). In these experiments seedlings were spray-treated with 1-50 mmol L^{-1} solutions of proline-derived tetramic acids and then monitored for fourteen days. At the end of this period, seedlings were classified into one of three possible classes: dead (directly exposed foliage died and no regrowth was observed after fourteen days), regrown

(directly exposed foliage had died completely but new foliage emerged within fourteen days) or unaffected (directly exposed foliage showed no symptoms or mild phytotoxicity only).

The importance of the acyl group is again clearly visible. In *A. retroflexus* (**Figure 5a**), the shortest side chain length, compound **11**, only caused significant mortality at 50 mmol L^{-1} (Fisher's exact test: $p = 0.029$). For compound **12,** 10 mmol L^{-1} suffices to cause 100% mortality, and for acyl chain lengths of six carbon atoms (compound **13**) or above, 5 mmol L^{-1} is sufficient. At chain lengths of nine carbon atoms (compound **15**) or longer, some mortality is seen even at 1 mmol L^{-1}, but this is not statistically significant.

The same pattern of increasing efficacy with longer side chains is visible in *S. media* (**Figure 5b**), although here regrowth occurs at intermediate concentrations. Interestingly, the *S. media* experiment suggests that increasing the length of the acyl side chain beyond ten carbon atoms (compound **7**) may not further enhance activity: TA12-Pro **7** causes significant mortality at 5 mmol L^{-1} (Fisher's exact test: $p = .048$), whereas compounds **15** and **16** at 5 mmol L^{-1} do not.

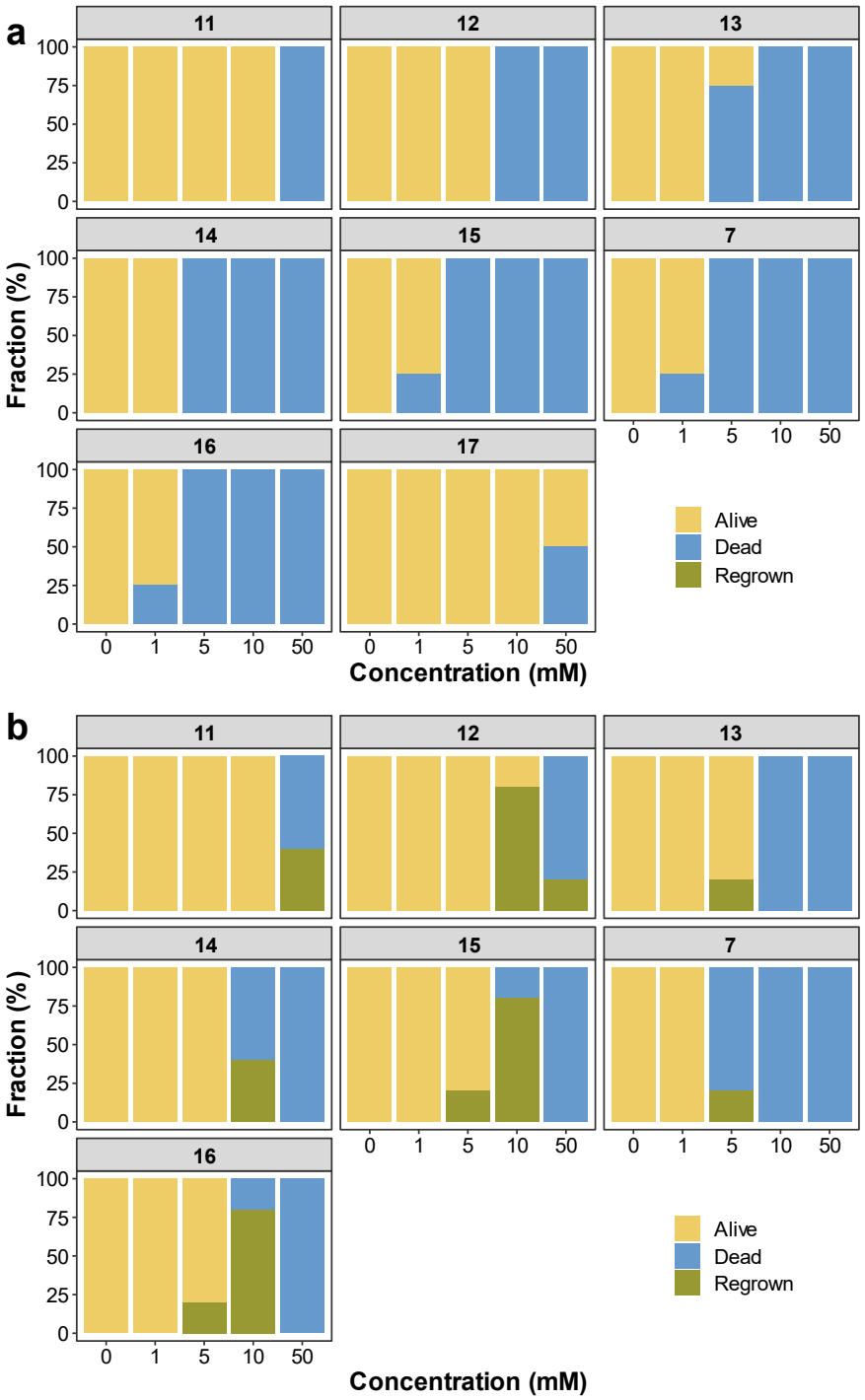


Figure 5: *Effect of varying side chain length on the herbicidal activity of proline-derived tetramic acids* ***7*** *and* ***11-17*** *against* Amaranthus retroflexus ***(a)*** *and* Stellaria media ***(b)***. *Seedlings were scored fourteen days after application as unaffected (directly exposed foliage survived with no or minor phytotoxicity, new growth visible), regrown (directly exposed foliage died but new growth emerged) or dead (directly exposed foliage died and no regrowth observed). N = 5.*

*Pre-emergent herbicidal activity of TA12-Pro **7***

After establishing the contact herbicidal activity of TA12-Pro ***7***, we also investigated whether ***7***, like DCMU, is effective as a pre-emergent herbicide against Amaranthus retroflexus (**Table 1**). A concentration of 5 mmol L^{-1} significantly delays emergence (0% emergence after five days, versus 20% in the blank control) but does not prevent emergence entirely (20% emergence after twelve days, versus 33% in the blank control). The highest tested concentration, 10 mmol L^{-1}, did however completely prevent emergence (0% after twelve days). DCMU showed considerably lower activity in this assay, with 15% emergence after 12 days even at 10 mmol L^{-1} most likely because our substrate is very rich in organic matter, which greatly reduces the efficacy of DCMU as a pre-emergent herbicide.[22]

Table 1: *Efficacy of TA12-Pro **7** as a pre-emergence herbicide against* Amaranthus retroflexus*. Immediately after sowing in 60 mL pots containing 50 mL of potting soil, seeds were covered with a further 10 ml of potting soil into which TA12-Pro **7**, DCMU or blank formulation were mixed to the target concentration. Emergence was then recorded at five, eight or twelve days after sowing (das). Emergence values shown are mean ± SEM, N = 4 (four pots with ten seeds each). Within each time point, treatments with different letters differ significantly according to Tukey's Honest Significant Difference test ($p < .05$).*

Treatment	**Concentration (mmol L^{-1})**	**Emergence 5 das (%)**		**Emergence 8 das (%)**		**Emergence 12 das (%)**	
Blank	0	20.0 ± 4.1	a	30.0 ± 4.1	a	32.5 ± 2.5	a
7	1	15.0 ± 2.9	ab	20.0 ± 4.1	ab	25.0 ± 4.1	ab
DCMU	1	12.5 ± 2.5	abc	25.0 ± 6.5	a	25.0 ± 6.1	a
7	5	0.0 ± 0.0	c	20.0 ± 7.1	ab	20.0 ± 7.1	ab
DCMU	5	10.0 ± 5.8	abc	22.5 ± 7.5	a	22.5 ± 7.5	ab
7	10	0.0 ± 0.0	c	0.0 ± 0.0	b	0.0 ± 0.0	b
DCMU	10	2.5 ± 2.5	bc	15.0 ± 2.9	ab	15.0 ± 2.9	ab

*Additional herbicidal selectivity assessment of TA12-Pro **7***

Selectivity is an important property of herbicides. Although the data shown previously in this manuscript do not indicate that TA12-Pro **7** is a selective herbicide, the effect of **7** on six additional crop species belonging to different plant families was evaluated in a small-scale spray test: sunflower (*Helianthus annuus* 'SY Bacardi'; Asteraceae), bean (*Phaseolus vulgaris* 'Sigma'; Fabaceae), tomato (*Solanum lycopersicum* 'Marmande'; Solanaceae), carrot (*Daucus carota* 'Evora'; Apiaceae), maize (*Zea mays* 'SY Fermin;' Poaceae) and wheat (*Triticum aestivum* 'SY Admiration'; Poaceae). Seedlings were sprayed once in the three-leaf stage and monitored for fourteen days.

As shown in **Table 2,** the positive control treatment (10 mmol L^{-1} DCMU) caused 100% mortality in almost all tested species. TA12-Pro **7** at the same concentration caused injury to all directly treated foliage, but plant mortality was lower: 75% in sunflower and carrot, and 0% in bean, tomato, wheat and maize. The regrowth shown by wheat and maize is in line with our results concerning the gramineous weeds *E. crus-galli* and *A. myosuroides*. The relatively high mortality seen in sunflower and carrot similarly agrees with our observations on the dicot weeds *A. retroflexus* and *S. media*. Bean and tomato phenotypically behaved more akin to what was observed in graminoids: they showed (near-) total death of the directly exposed foliage but did show signs of regrowth. These results show that there are differences in the relative susceptibility of plant species to TA12-Pro **7**, but that it is still clearly a non-selective herbicide.

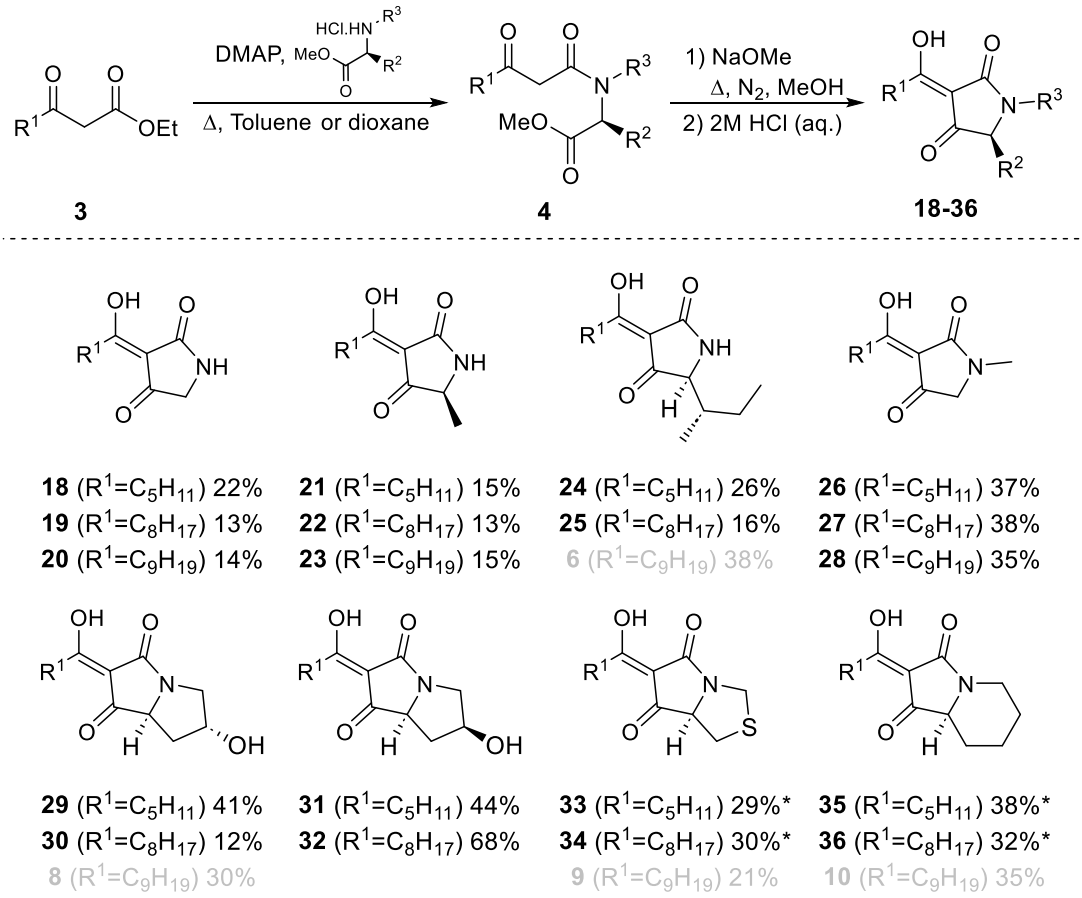
Table 2: *Selectivity of TA12-Pro* ***7*** *towards a panel of economically important crop species: sunflower (*Helianthus annuus*), bean (*Phaseolus vulgaris*), tomato (*Solanum lycopersicum*), carrot (*Daucus carota*), maize (*Zea mays*) and wheat (*Triticum aestivum*). The percentage of injured and dead seedlings is shown two weeks after spraying with a formulation control or a 10 mmol L^{-1} solution of DCMU or TA12-Pro* ***7****. 'Injured' refers to visible necrosis or death of directly exposed tissues but without plant death. N = 4.*

	Formulation		**TA12-Pro 7**		**DCMU**	
	Injured (%)	Dead (%)	Injured (%)	Dead (%)	Injured (%)	Dead (%)
H. annuus	0	0	25	75	0	100
P. vulgaris	0	0	100	0	0	100
S. lycopersicum	0	0	100	0	0	100
D. carota	0	0	25	75	0	100
Z. mays	0	0	100	0	0	100
T. aestivum	0	0	100	0	25	75

Screening of additional 3-ATA analogs for SAR investigation

To expand the SAR analysis, a second library of 3-ATAs containing eight different AA moieties was synthesized. For the sake of comparison some previously tested ones were again included, whereas several new ones were also introduced. The final set consisted of derivatives of glycine, sarcosine, L-alanine, L-isoleucine, L-pipecolic acid, L-proline, L-thiaproline, (R)-hydroxyproline and (S)-hydroxyproline, each synthesized with acyl chain lengths of 6, 9 and 10 carbon atoms (comparable with compounds **13**, **15** and TA12-Pro **7**). Derivatives with these chain lengths showed good efficacy in the initial spraying experiments with the L-proline-derived 3-ATAs and were thus chosen for this expanded screening. By varying both side chain length and AA moiety in the same experiment, we could evaluate whether the pattern seen with the L-proline-derived 3-ATAs holds generally or varies between AA moieties. The novel compounds could all be synthesized straightforwardly via the same methodology, yielding the targeted 3-ATAs **18**-**36** (see

Scheme 3). It is interesting to note, however, that some of the reactions performed with secondary amino acids thiaproline and pipecolic acid yielded the target tetramic acid already in the first reaction step, thus making the Lacey-Dieckmann rearrangement with sodium methoxide obsolete. To the best of our knowledge, this type of reactivity has not yet been described in literature and thus should be investigated further to explain why this does not take place with e.g. L-proline or sarcosine. Of these analogues, compounds **18**[23], **20**[23], **23**[24], **26**[25] and **28**[25] have been described in literature before.



*Scheme 3: Synthesis of 3-ATAs **18-36** with different amino acid moieties and acyl chain lengths of 6, 9 and 10 carbon atoms. The compounds in gray were already synthesized in Scheme 1 but are again included in this library. *Reaction showed conversion to the tetramic acid already in the first step.*

Against *A. retroflexus*, all tested 3-ATAs achieved a significant reduction in F_v/F_m at a concentration of 10 mmol L^{-1}, except for the (S)-hydroxyproline analogs **31** and **32** (**Figure 6a**). The glycine (**18-20**), L-alanine (**21-23**) and (R)-hydroxyproline analogs (**29**, **30** and **8**) also showed limited efficacy. All other

tested amino acid derivatives – L-proline (**7**), L-thiaproline (**33**, **34** and **9**), sarcosine (**26-28**) and L-pipecolic acid (**35**, **36** and **10**) - achieved a reduction in F_v/F_m that was statistically identical both to each other and to DCMU. A similar division in two activity classes is even more clearly visible with *A. myosuroides*, where derivatives of glycine (**18**, **19**), L-alanine (**21-23**) and both (R)- and (S)-hydroxyproline (**8** and **29**-**32**) do not show statistically significant activity, whereas the other AA moieties all perform at least on par with DCMU (**Figure 6b**). These results indicate that the use of a secondary amino acid is necessary for a good activity, since sarcosine derivatives perform on par with the proline-derived tetramic acids. Contrastingly, a hydroxyl group on the proline-ring leads to a decrease in activity, even though the thiazolidine moiety in L-thiaproline-derivatives is tolerated for good activity. This leads us to believe that the additional hydroxyl group changes the overall polarity or conformation of the molecule, thereby decreasing the activity of the compound.

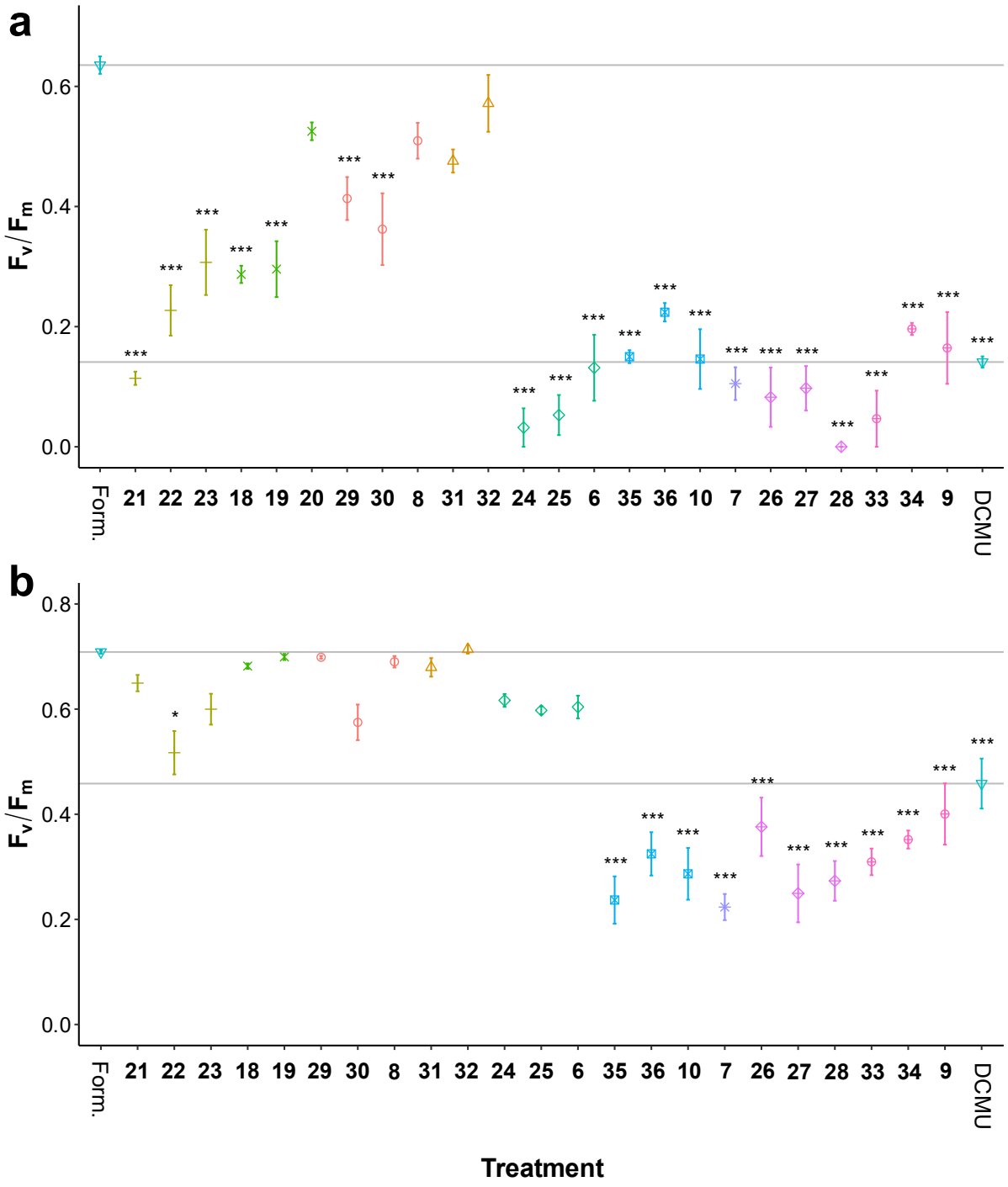


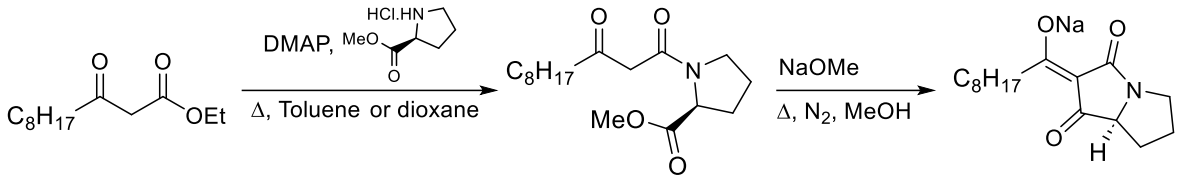
Figure 6: *Effect of 3-ATAs* ***6-10*** *and* ***18-36*** *with varying amino acid moieties and side chain lengths on seedlings of* Amaranthus retroflexus ***(a)*** *and* Alopecurus myosuroides ***(b)***. *F_v/F_m was measured 72 hours after spraying until run-off with a 10 mmol L^{-1} solution. Error bars indicate mean ± SEM, N = 4. Asterisks indicate significant difference from the formulation control (indicated with 'Form.') according to Tukey's Honest Significant Difference test (*: p < .05); **: p < .01; ***: p < .001). The upper and*

lower horizontal gray lines correspond to the mean of the formulation negative control and DCMU positive control respectively as an aid to the reader. Compounds with the same amino acid moiety share the same color and symbol.

Regarding the length of the acyl side chain, our data show no differences between 6, 9 and 10 carbon atoms in the acyl chains across all tested compounds (C6 *vs*. C9: -21%, p = .68; C6 *vs*. C10: -10%, p = .97, C9 *vs*. C10: +14%, p = .90), although for some individual amino acid derivatives differences between chain lengths were observed. This indicates there might be amino acid-specific interactions with side chain length, but given the relatively small panel of compounds and sample sized used in this study further research is needed to confirm whether this is indeed the case.

The acidic proton of 3-ATAs is required for activity

The most important part of the studied structures is the 'triketone' or β,β'-dioxoamide moiety. It is less straightforward to synthesize analogues of this functional group, although during a replication of the synthesis of compound **15**, we managed to isolate a small amount of the corresponding sodium salt by purifying one of the waste fractions. We confirmed the structure via the NMR-characterization and physicochemical behavior of the molecule, as this salt is readily soluble in water, in sharp contrast to its corresponding acid, compound **15**. Of course, by omitting the neutralization step in the aforementioned procedure, this novel sodium salt **37** could be isolated in a 56% yield. This seemed a suitable product to investigate the role of the acidic proton of 3-ATAs in their herbicidal activity. When applied against *A. retroflexus*, the salt shows a complete loss of phytotoxicity (**Figure 7**), thereby confirming the crucial role of the acidic proton. This crucial role is also confirmed in a study by the Stallforth group, who have shown that a methylated compound **14** shows no amoebicidal nor antiproliferative activity, in contrast to compound **14** itself.[7] Therefore, their results also confirm the necessity of this acidic proton for the bioactivity of these structures.



*Scheme 4: Adapted synthesis for the isolation of the sodium salt **37**.*

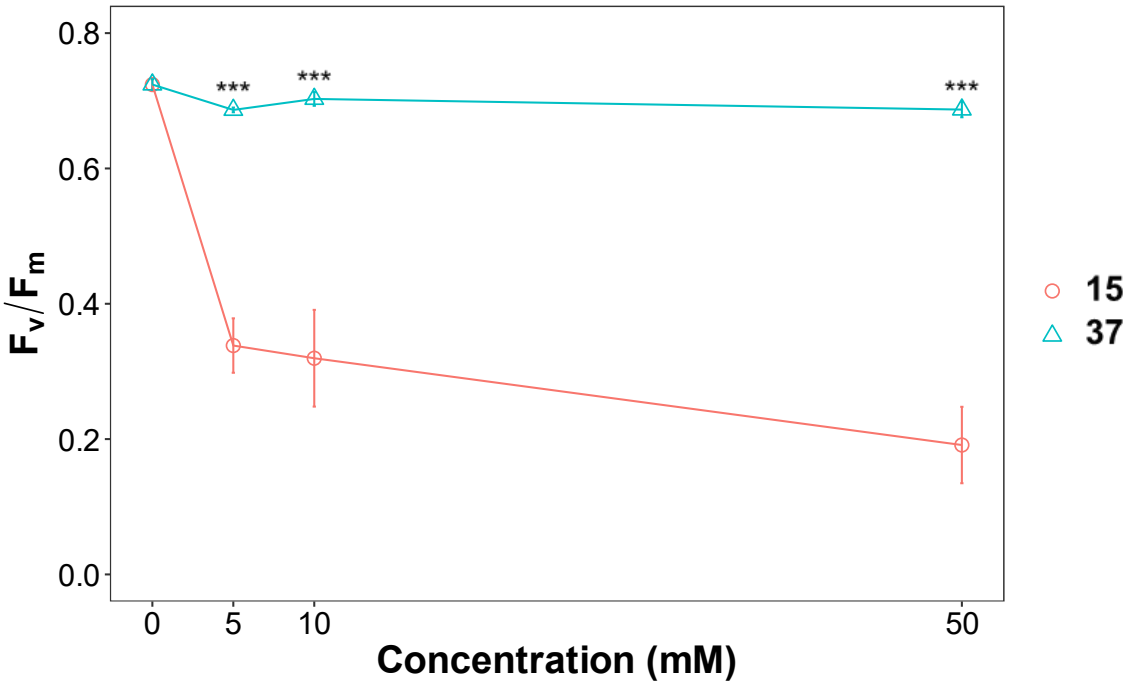


Figure 7:** Loss of herbicidal activity against* A. retroflexus *of sodium salt **37** compared to the corresponding acid **15**. F_v/F_m was measured 72 hours after spraying until run-off. Error bars indicate mean ± SEM, N = 4. Asterisks indicate significant difference from the formulation control according to Tukey's Honest Significant Difference test (: p < .0001).*

*Leaf disk assays indicate that the herbicidal activity of TA12-Pro **7** is at least partially explained by light-independent cell lysis*

While a thorough evaluation of the mechanism of action of 3-ATAs falls outside the scope of this study, we were intrigued by previous observations in algae showing that TA12 (**2)** induced cell lysis in addition to PSII inhibition, an activity not displayed by DCMU.[9] Additionally, previous reports of the Stallforth group have identified TA12-Pro **7** as part of a set of 3-ATAs that are produced by a *Pseudomonas fluorescens* strain as amoebicides.[6] In follow-up research, the same authors also found that these 3-ATAs induce cell death in three strains of Gram-positive bacteria, likely through protonophore activity.[7] Since these results point towards possibly cellular toxicity through means other than PSII inhibition, we attempted to assess whether PSII activity is indeed the principal or even only herbicidal mechanism of 3-ATAs in plants through a series of *in vitro* leaf disk assays.

First, we tested the light dependence of the herbicidal activity of TA12-Pro **7**: tomato leaf disks were exposed to either **7** or DCMU and subsequently incubated either in complete darkness or under ambient light conditions. After 24 hours and 48 hours, the F_v/F_m of these leaf disks was measured. Since this requires exposing leaf disks to a brief but intense light pulse, separate batches of leaf disks were used at 24 and 48 hours. Our data show that the effect of DCMU was greatly diminished by dark incubation (**Figure 8**), likely because far fewer ROS are formed upon PSII inhibition when plants are not exposed to light. Treatment with TA12-Pro **7**, meanwhile, showed a smaller reduction in F_v/F_m after 24 hours of exposure in the dark compared to light incubation but dropped to the same F_v/F_m after 48 hours both in the dark and light incubation.

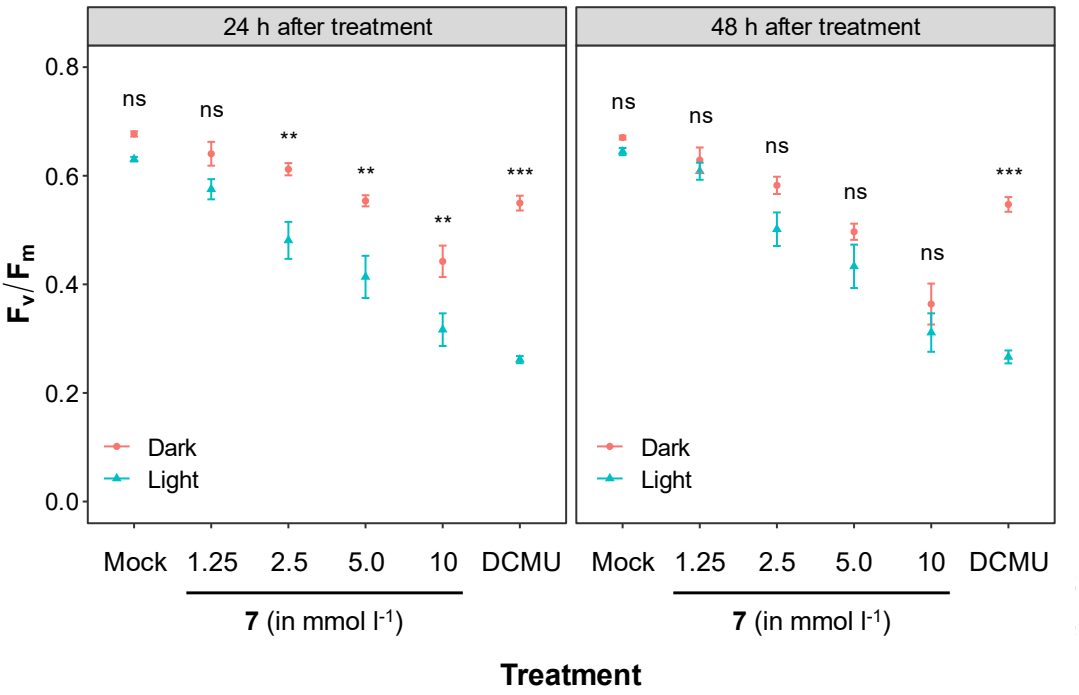


Figure 8: *Differential effect of TA12-Pro* ***7*** *and DCMU on F_v/F_m in light- and dark-incubated* Solanum lycopersicum *leaf disks. Error bars indicate mean ± SEM. N = 4. Asterisks indicate significant difference between light and dark incubation according to Tukey's Honest Significant Difference test (ns: p > .05; *: p < .05; **: p < .01; ***: p < .001).*

We observed that leaf disks exposed to TA12-Pro **7** begin to show intense brown discoloration within 48 hours after exposure, whereas no discoloration is visible in DCMU-treated leaf disks – a phenomenon previously observed in leaves exposed to tenuazonic acid and which was found to co-localize with areas of cell lysis.[8] This brown coloration persists even when the leaf disks are cleared in hot ethanol, which excludes the possibility that the brown coloration is caused by chlorophyll degradation or pigmentation (**Figure 9**).

Staining TA12-Pro- or DCMU-treated leaf disks with 3,3'-diaminobenzidine (DAB), a common stain to visualize hydrogen peroxide accumulation,[26] reveals another marked difference between TA12-Pro **7** and DCMU. Whereas DCMU-treated leaf disks show faint, sporadically scattered staining throughout the leaf disk, TA12-Pro-treated leaf disks show highly intense staining in and around the discolored areas that form

at and around the droplet application sites. Interestingly, the most intense brown staining occurs immediately outside the discolored area rather than inside it (**Figure 9**). Again, H_2O_2 accumulation was also previously observed in leaves exposed to tenuazonic acid, but not DCMU, and found to correspond with areas of cell lysis.[8] Together, these results show that TA12-Pro **7** causes both inhibition of photosynthesis and cell lysis, and our dark incubation experiments indicate that cell lysis alone is sufficient to cause cell death.

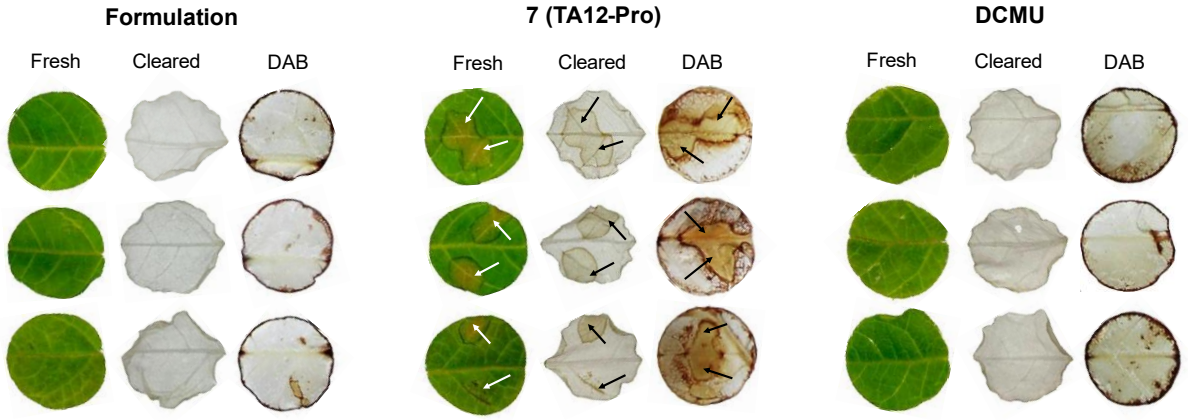


Figure 9: *Representative images of* Solanum lycopersicum *leaf disks sampled 72 hours after exposure to a formulation control, 10 mmol L^{-1} of TA12-Pro **7** or 10 mmol L^{-1} of DCMU. Pictures are shown of freshly collected leaf disks (left), cleared leaf disks (center) and leaf disks stained with 3,3'-diaminobenzidine (DAB) and then cleared. Arrows indicate the discolored sites observed at TA12-Pro **7** application sites. Images of fresh and cleared leaf disks were taken from the same batch of disks, the DAB-stained disks came from a separate batch.*

*Validation of TA12-Pro **7** in field conditions*

As a final validation of the efficacy of 3-ATAs, the efficacy of TA12-Pro **7** was evaluated in a field trial. A 36 m^2 false seed bed was prepared in an arable field at the Bottelare experimental farm (Merelbeke, Belgium) to achieve a uniform weed stand, the center of which was divided into 1.5 m^2 subplots. On the day of the trial, the individual plants present in a randomly selected 40 x 40 cm square in each subplot were

surveyed to get an insight into the precise composition of the weed population of the trial field. There was little variation in species composition between subplots; across all plots the weed population consisted of 68% *Galinsoga* sp., 22% *Coronopus* sp., 8% *Spergula arvensis* and 2% minor species (*Viola arvensis*, *Chenopodium album*, *Lamium purpureum*, *Echinochloa crus-galli*, *Polygonum aviculare*, *Persicaria maculosa*, *Stellaria media*, *Cirsium arvense*, *Senecio* sp.). Four subplots were used per treatment. The commercial herbicide Beloukha® (Certis-Belchim, active ingredient: pelargonic acid) applied according to label instructions was used as a positive control, whereas untreated and formulation-only checks were used as negative controls. TA12-Pro **7** was applied as 10 and 100 mmol L^{-1} spraying solutions with a backpack sprayer at a rate of 300 L ha^{-1}, corresponding to dose rates of 0.88 kg ha^{-1} (3 mol ha^{-1}) and 8.8 kg ha^{-1} (30 mol ha^{-1}) respectively. RGB images were taken using drone-mounted cameras (**Figure 10**), after which the percentage of pixels showing living vegetation was calculated. Our results show that, in contrast to indoor growth chamber experiments, a 10 mmol L^{-1} TA12-Pro **7** solution is not effective at killing weeds in open field conditions. However, a 100 mmol L^{-1} solution achieved equivalent weed control as Beloukha® despite a significantly lower active ingredient concentration (**Table 3**): 30 mol ha^{-1} versus 69 mol ha^{-1}. Neither product showed appreciable residual efficacy, as significant regrowth had already occurred within seven days after treatment. Although these results are promising, the small scale of the trial and the overwhelmingly broadleaved resident weed population (> 99.5%) mean that further trials at different sites with more diverse weed populations and optimalisation of the formulation of the active ingredient are required to better understand the potential and limitations of 3-ATAs as herbicides.

Table 3: *Efficacy of TA12-Pro **7** at weed control in field conditions. Weed reduction is defined as the reduction in the fraction of green pixels in areal RGB images taken four and seven days after application compared to the moment of herbicide application. N = 4 (four 1.5 m^2 subplots used per treatment). Treatments with different letters differ significantly according to Tukey's Honest Significant Difference test ($p < .05$). Pelargonic acid was used in the form of the commercial herbicide Beloukha® (Certis-Belchim, 680 g L^{-1} EC).*

Treatment	**% weed reduction after 4 days (± SEM)**		**% weed reduction after 7 days (± SEM)**	
Untreated	0 ± 0	a	0 ± 0	a
Formulation control	0 ± 0	a	0 ± 0	a
Pelargonic acid 10.9 kg ha^{-1} (69 mol ha^{-1})	81 ± 14	b	43 ± 5	b
TA12-Pro **7** 0.88 kg ha^{-1} (3 mol ha^{-1})	3 ± 1	a	3 ± 1	a
TA12-Pro **7** 8.8 kg ha^{-1} (30 mol ha^{-1})	88 ± 3	b	48 ± 2	b

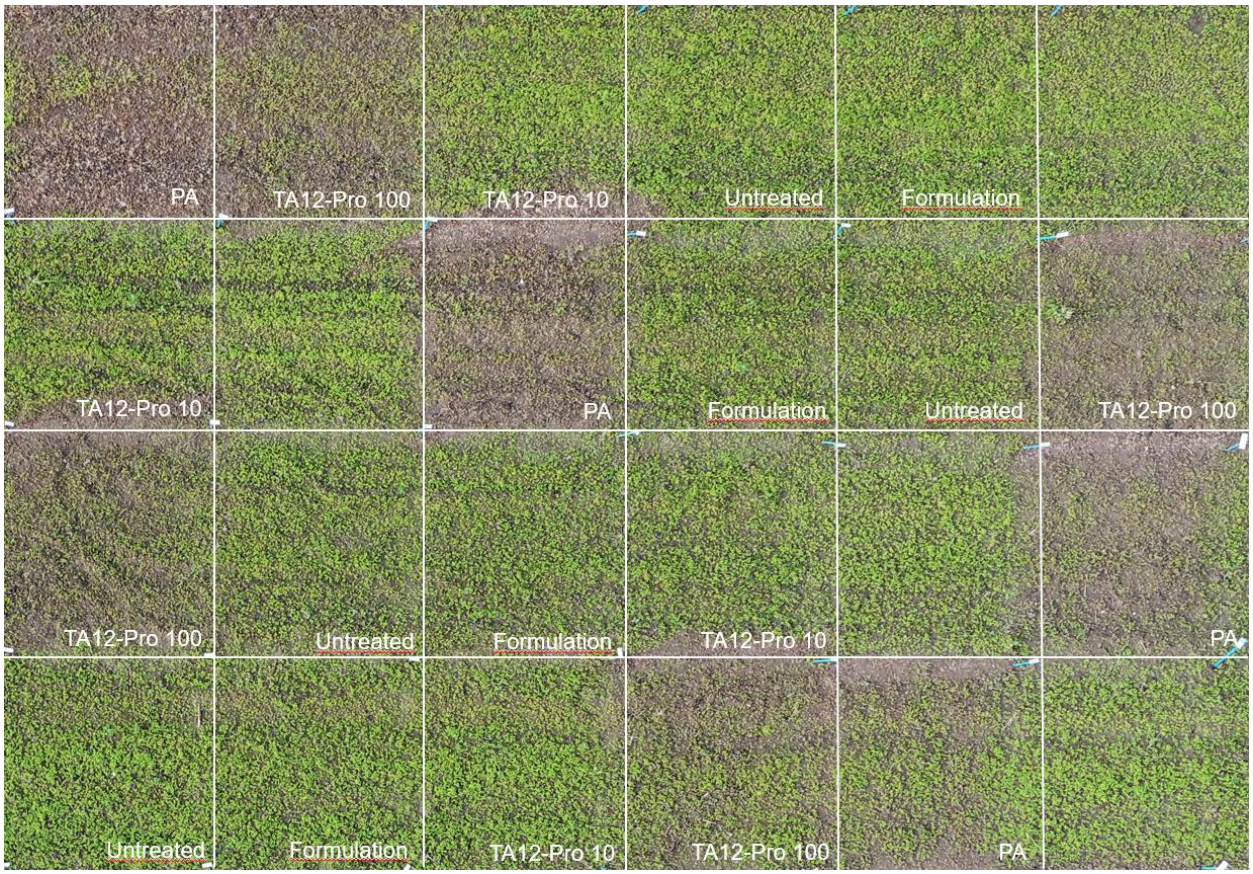


Figure 10: *Efficacy of TA12-Pro **7** in field conditions. Photograph was taken with a drone-mounted RGB camera four days after treatment. Abbreviations: PA = pelargonic acid (Beloukha®, Certis-Belchim, 10.9 kg ha^{-1}), TA12-Pro 10 = 10 mmol L^{-1} spraying solution TA12-Pro **7** (0.88 kg ha^{-1}), TA12-Pro 100 = 100 mmol L^{-1}spraying solution TA12-Pro **7** (8.8 kg ha^{-1}). Each subplot has a 1.5 m^2 surface area.*

*Preliminary ecotoxicological assessment of TA12-Pro **7***

As a first step towards understanding the ecotoxicological potential of TA12-Pro **7**, a preliminary assessment of the acute toxicity and biodegradability of TA12-Pro **7** in freshwater settings was conducted. Acute toxicity tests were performed with the indicator organisms *Raphidocelis subcapitata* (a green algae, formerly known as *Pseudokirchneriella subcapitata*), *Daphnia magna* (a daphnid) and *Lemna minor* (an aquatic plant). In addition, biodegradation by freshwater microbes was also evaluated. All tests were conducted as prescribed in the respective OECD guidelines.[27–29]

EC_{50} values for *R. subcapitata* and *L. minor* were calculated as 7.15 and 1.83 mg L^{-1} respectively. For *D. magna,* the EC_{50} value lies between 1 and 10 mg L^{-1} but could not be precisely quantified due to a lack of intermediate concentrations in the assay. Based on these EC_{50} values, European Union Regulation (EC) No 1272/2008 would not define TA12-Pro **7** as acutely toxic to any of the three tested indicator organisms.[30] Biodegradation of TA12-Pro **7** after four weeks was 40%, which is defined as *inherently* but not *readily* biodegradable under OECD guidelines.[31]

Structure-activity relationships of 3-ATAs

We demonstrated a flexible and readily accessible methodology for the synthesis of 3-ATAs and used it to synthesize libraries of analogues to enable thorough exploration of the herbicidal activity of this scaffold. First, we established that the algicidal activity of TA12 (**2)** could be transferred to herbicidal activity through an *in vitro* leaf disk assay and spray tests with agronomically important weed species. Subsequently, we showed that the presence of a medium or long chain acyl group (more than six carbon atoms in the acyl chain) greatly enhances activity relative to shorter side chains. For the amino acid moiety, apolar secondary amino acid derivatives showed higher activity than other tested amino acid moieties. Study of the isolated sodium salt **37** allowed us to show that the presence of the acidic proton of the β,β'-dioxoamide moiety is essential for the herbicidal activity of the 3-ATA (**Figure 11**). With regards to the

evaluated weeds, high mortality was achieved against broadleaf seedlings (*A. retroflexus* and *S. media*), but gramineous weeds (*E. crus-galli* and *A. myosuroides)* invariably showed regrowth. Herbicidal activity of TA12-Pro **7** was also demonstrated against broadleaf weeds in field conditions. Finally, a preliminary assessment of the ecotoxicology revealed that TA12-Pro **7**, with promising herbicidal activity, is not acutely toxic for the evaluated organisms and can be classified as inherently biodegradable.

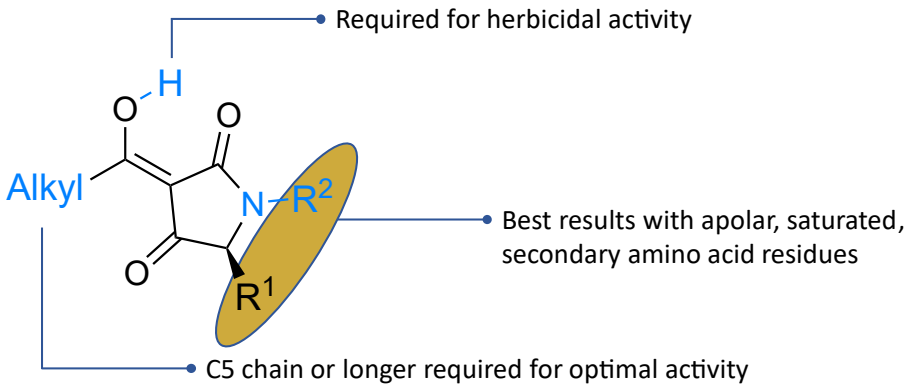


Figure 11: *Summary of the structure-activity relationships of the demonstrated 3-ATAs in this study.*

Preliminary mechanistic studies furthermore showed that, in contrast to treatments with DCMU, 3-ATAs retain activity in the absence of light and induce brown discoloration and strong hydrogen peroxide accumulation in exposed tissues. Together, this points towards cell lysis as an additional mode of action in addition to the previously demonstrated photosystem II inhibition.

Together, our research sheds new light on the chemistry and phytotoxicity of amino acid-derived 3-acyltetramic acids and shows the potential of this scaffold for herbicide development. Further research will be required, however, to further enhance its activity, to elucidate its mechanism(s) of action and to understand its (eco)toxicological properties and environmental fate.

Supporting Information

The Supporting Information contains materials and methods for the chemical synthesis and biological evaluation and ^{1}H, ^{13}C NMR, IR and MS spectra used for the characterization of the final compounds, as well as the raw data underlying the graphs and tables shown in this manuscript.

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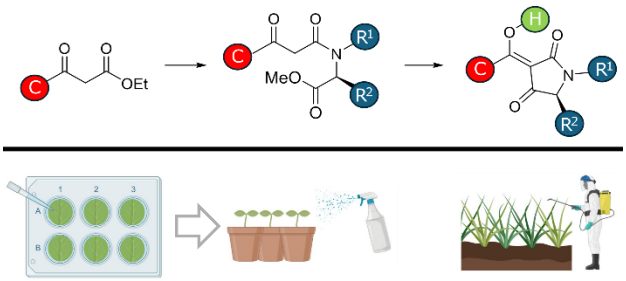
601 

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