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Host Marking Pheromone (HMP) in the Mexican Fruit Fly *Anastrepha ludens*

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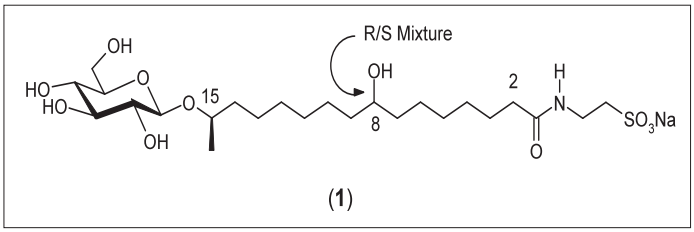
Dedicated to Professor Daniel Belluš on the occasion of his 70th birthday

Abstract: Host marking pheromones (HMPs) are used by insects to mark hosts (usually a fruit) where they have already laid eggs. The compounds serve as a deterrent to conspecifics avoiding over-infestation of hosts (*i.e.* repeated egg-laying into an already occupied/used host). If these HMPs are sprayed onto commercially valuable fruit they act as deterrents preventing attack by females interested in laying eggs into the valuable commodity. Having no insecticidal or toxic properties, and being natural products (or close derivatives thereof) they could be used as fruit sprays to replace insecticides, or in combination with other products to improve efficacy. This review discusses the isolation, and synthesis of the HMP of the Mexican fruit fly *Anastrepha ludens* a feared pest of citrus and mangos in Mexico and Central America. This compound is also recognized by females of other pestiferous species in the same genus *Anastrepha* distributed from the Southern USA to Northern Argentina, including many Caribbean Islands. The synthetic HMP was shown to exhibit strong electrophysiological activity against *A. ludens* and excellent interspecies cross recognition with other *Anastrepha* species. Behavioural tests confirmed the HMP deterring effect of the synthetic natural product. Further studies enabled us to drastically simplify the structure of the HMP and obtain a derivative, which we named *Anastrephamide*, which shows HMP deterring effects very similar to the natural product in laboratory and field tests. The potential use of such HMP derivatives in a crop protection scenario is briefly discussed.

Keywords: *Anastrephamide* · Crop protection · Fruit flies · Host marking pheromones · Natural products

1. The Importance of Host Marking Pheromones (HMPs)

Host marking pheromones (HMPs) are used by certain insects to mark hosts where they have already laid eggs. In the case of *true fruit flies* (family *Tephritidae* as opposed to *Drosophilidae*), this is achieved by dragging its aculeus (tip of ovipositor), over the surface of the fruit after an egg-laying bout. The HMPs are also found in the fly faeces.[1]



(**1**)

Fig. 1. HMP of the European cherry fruit fly, *Rhagoletis cerasi.*

The compounds serve as a deterrent to conspecifics, thus avoiding over-infestation of hosts (*i.e.* repeated egg-laying into an already occupied/used host).[2] If these HMPs are sprayed onto commercially valuable fruit they act as deterrents preventing attack by females interested in laying eggs into the valuable commodity. Having no insecticidal or toxic properties, and being natural products (or close derivatives thereof), they could be used as fruit sprays to replace insecticides, or in combination with other products to improve efficacy. There have already been successful applications of such a crop protection strategy reported in the literature. In particular, Ciba Geigy (now Syngenta) in collaboration with the Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture, Wädenswil, Switzerland (now Swiss Federal Research Station Agroscope Changins-Wädenswil ACW) were able to identify the HMP of the European cherry fruit fly, *Rhagoletis cerasi* by a combination of bioassay-guided isolation from methanol extracts of the fly faeces, and total synthesis.[3] The natural product ((**1**), Fig. 1) when applied to cherry orchards, showed high levels (up to 98%) of activity (*i.e.* reduced fruit infestation).[4]

So why has this seemingly environmentally benign method of pest control not been further pursued? Are HMPs confined to the European cherry fruit fly? Clearly one would not expect this to be the case, and HMPs have indeed been found in about 100 species from five insect orders.[5] In the most cases, the structures of the HMPs remain unknown often due to the difficulty of isolation and structure determination, and consequently there has been very little undertaken to look at these HMPs as crop protection agents.[6]

In this article we briefly describe our findings concerning the HMP of another fruit fly pest, namely the Mexican fruit fly *Anastrepha ludens*, a feared pest of citrus and mangos in Mexico and Central America. A detailed account of isolation, syn-

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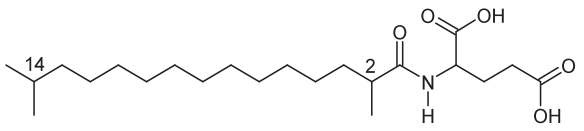
theses, and biological/field testing of compounds described here have appeared in the patent[7] and mainstream literature,[8,9] but the work has never been reviewed in terms of chemistry, biological activity and structure–activity relationships.



(**2**)
N-(2,14-dimethyl-1-oxopentadecyl)-Glutamic acid,

Fig. 2. Structure of the HMP of *Anastrepha ludens*.

2. Isolation and Structure of *Anastrepha ludens* HMP

Several species of *Anastrepha* are important pests in the Americas. For example, commercial crops including guava, oranges, grapefruit, mango, tropical plums, mammee apple, chico zapote are infested by various *Anastrepha* species such as *A. fraterculus, A. grandis, A. ludens, A. obliqua* and *A. serpentina* throughout South America and southern USA.[10]

It has been observed that this fruit fly species exhibited similar behavioural activity to the European cherry fruit fly (*Rhagoletis cerasi*), also in terms of oviposition.[2] Thus, it could be demonstrated that methanol extracts obtained from the faeces of one of the species of these fruit flies (*Anastrepha ludens*) showed HMP deterring activity, not only to *Anastrepha ludens*, but with complete interspecies cross recognition to *A. obliqua* from the *fraterculus* species group and *A. serpentina*.[8] The next step was thus clear: Isolate the active principle from the faeces methanol extracts, determine the structure, test the biological activity, and simplify the molecule whilst retaining the HMP deterring properties.

The first part of this story is beyond the scope of this review, but clearly bioassay-guided isolation of the active principle from the methanol extract of *Anastrepha ludens* faeces was key. In short, the methanol concentrate was subjected to HPLC purification and the fractions analysed using electrophysiology. This test is based upon the fact that chemoreceptor sensilla (chemosensilla) containing a receptor cell sensitive to the HMP have been identified on the distal ventrolateral portions of the second, third and fourth tarsomeres of each leg (lowermost part of leg with which the insect contacts the surfaces it walks over).[11] These sensilla are known as 'D'-chemosensilla. Usually, every tarsi has six of these sensilla, and as insects have six legs, there are a total of 36 'D'-chemosensilla potentially responding to the HMP. D-chemsensilla are thus extremely useful when trying to determine the response by the insect to the HMP during analysis of HPLC fractions. Using an electrophysiological test, female flies are decapitated and mounted ventral side up to expose the D-sensilla which are humidified continuously with a water-saturated air stream. Using electrophysiological techniques known as 'tip recordings' described in detail by Dethier[12] and Städler,[13] the chemosensillum is stimulated and the ensuing spikes (action potentials) are analyzed. Spike activity varies according to the level of response by the insect and can be used to distinguish between active and inactive HPLC fractions of the HMP. Using this bioassay, we were eventually able to isolate 5 mg of a single compound with very high electrophysiological activity from 167 g of fly faeces. However, since a positive electrophysiological response does not always translate into a positive behavioural response, additional bioassays had to be performed in the lab. When a HMP comes into contact with its chemosensilla, a female fly responds by shaking its legs, cleaning them and walking or flying away. Since females mark the fruit surface with HMP after laying eggs by dragging its aculeus (tip of ovipositor), when the same female or another one contacts a marked surface (fruit), it walks or flies to another fruit or a leaf. This behavioral response is measured by, for example, counting the total number of fruit visited by a female per unit time or the amount of time a female spends on top of a fruit. Instead of using fresh fruit as oviposition substrates, we utilized green 2.5 cm diameter agar spheres wrapped in parafilm. The flies were continuously observed, and their landings, oviposition attempts or successful ovipositions on treated or untreated spheres, recorded.[4]

With information on successful ovipositions, a discrimination coefficient (DC) for the flies to the artificial fruits could be calculated as described by Boller and Hurter.[14] The DC can vary between –100 and +100. A DC of –100 indicates that all eggs were laid into treated oviposition substrates. No difference between test substance and control would produce a DC of zero and hence no deterrent effect was achieved. A DC of +100 indicates that no eggs were laid into treated oviposition substrates and hence an absolute deterrent effect was achieved.

The 5 mg of isolated material showed a high deterring effect (90%) in the behavioural assay, confirming that the electrophysiological activity was related to a HMP effect.

With the pure HMP in hand, structure elucidation could be undertaken. Would this be a complicated natural product? Would it be possible to simplify this natural product and retain activity? We were very pleasantly surprised, when the structural elucidation[7] presented a relatively simple looking compound containing an isopalmitc fatty acid chain substituted by methyl at the 2-position and coupled to a glutamic acid (**2**, Fig. 2), apparently as a single diastereomer.[15]

It is immediately apparent that this molecule is considerably simpler than that of the European cherry fruit fly shown in Fig. 1. However, some work had to be invested in the first instance to determine the absolute stereochemistry of the natural product, as it contained two stereocentres and thus could have been one of four possible diastereomers: The four possible diastereomers can be described as (*R*)-L, (*S*)-L, (*R*)-D, (*S*)-D, in which *R* or *S* describe the chirality at the 2-methyl position in the fatty acid chain, and L or D indicate the chirality of the glutamic acid (Fig. 3).

The configuration of the amino acid was determined by hydrolysing the natural product under acidic conditions, and the glutamic acid portion derivatised to N-trifluoroacetyl glutamic acid isopropyl ester. This was then subjected to GC analysis on a chiral column (Chirasil-L-Val). Under these conditions,[16] the L-isomer showed a retention time of 25.6 min., the D-isomer 24.2 min. based on the synthetic compounds. The sample of the derivatised natural product consisted of 21% D-glutamic acid and 79% L-glutamic acid which was confirmed by co-injection authentic samples. This result was surprising to us as the ^{1}H and ^{13}C NMR indicated the presence of only one diastereomer, and thus we suspected that partial epimerisation of the amino acid occurred under the conditions of amide hydrolysis. Therefore, we addressed the question of the absolute configuration of the natural product by total synthesis of all four diastereomers. Comparison of the spectral and biological properties would enable us to assign the absolute configuration of *A. ludens* HMP (**2**), and also give us initial results on the importance of stereochemistry upon biological activity.

3. Synthesis and Absolute Configuration of *A. ludens* HMP

To achieve the synthesis of all four diastereomers, we required a stereoselec-

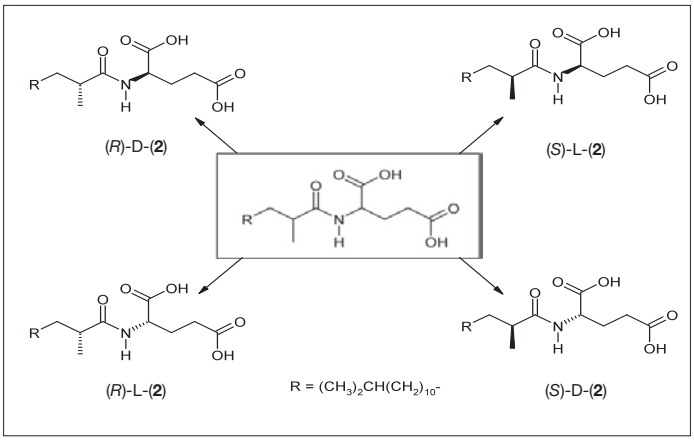
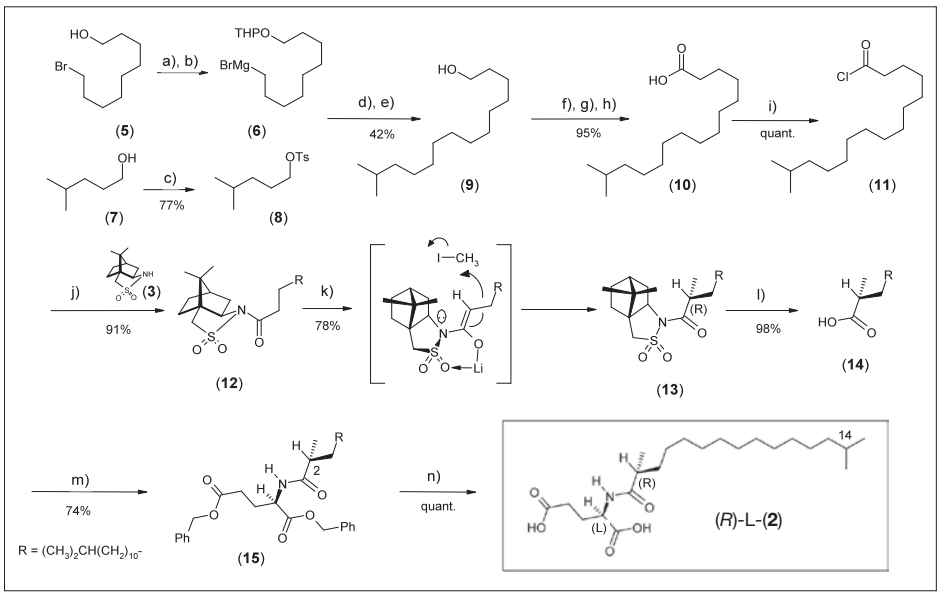
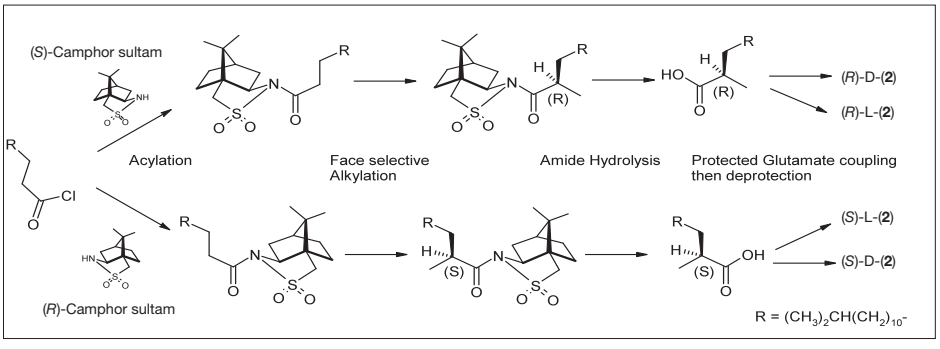


Fig. 3. Possible diastereomers of the HMP of *A. ludens* (**2**).



Scheme 1. Reagents and conditions: a) 2,3-Dihydropyrane, cat. p-TsOH, CH_2Cl_2, r.t. b) Mg, THF. c) TsCl, Et_3N,cat. DMAP, CH_2Cl_2, r.t. d) Li_2CuCl_4 (0.02 equiv.), add (**8**), THF, –78 °C to r.t. e) p-TsOH, MeOH, r.t. f) TsCl, Et_3N,cat. DMAP, CH_2Cl_2, r.t. g) KCN, DMSO, 80 °C. h) KOH, EtOH, reflux. i) $SOCl_2$, cat.. DMF, r.t. j) NaH, Toluene, r.t. k) n-BuLi, THF:DMPU (4:1), –78 °C, then add MeI –78 °C to r.t. l) LiOH, H_2O_2 THF:H_2O (9:1). m) L-Glutamic acid dibenzyl ester 4-toluenesulfonate, EDC, Et_3N, cat. DMAP, CH_2Cl_2. n) HCO_2H (88% in H_2O):MeOH, (4:1), Pd/C (10%).



Scheme 2. Synthesis of the diastereomers of the HMP of *A. ludens* (**2**).

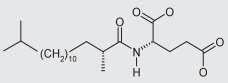
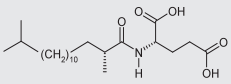
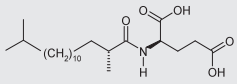
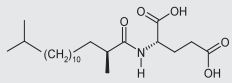
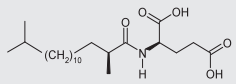
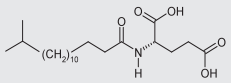
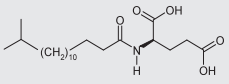
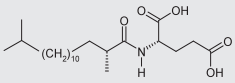
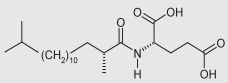
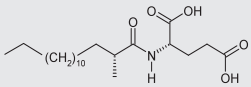
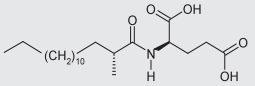
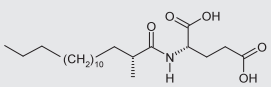
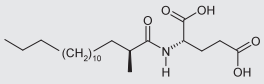
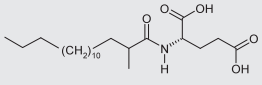
tive synthesis of the 2-methyl isopalmitic acid side chain with known absolute configuration, coupling of this to a suitably protected form of either L-, or D-glutamic acid, and subsequent deprotection without epimerisation of the chiral centres. To prepare the 2-methyl isopalmitic of known absolute configuration, we chose to attach the Oppolzer camphor sultam (**3**) to isopalmitic acid (**10**), carry out a stereoselective α-amide alkylation, and then remove the chiral auxiliary. The choice of the camphor sultam (**3**) for confident prediction of the stereochemical outcome of the alkylation is due to attack of the alkyl halide to the least hindered face of the lithium chelated enolate.[16,17] The synthesis is illustrated for the (*R*)-L-(**2**) in Scheme 1, which also shows our route for the preparation of isopalmitic acid for completeness.

Thus, 9-bromo-1-nonanol (**5**) was protected as its THP ether and converted to the Grignard (**6**). This was coupled to the branched chain tosylate (**8**) in THF in the presence of lithium chloride-cupric chloride catalyst,[18] followed by THP-deprotection to give the branched chain alcohol (**9**). The alcohol (**9**) was tosylated, converted to the cyanide, which after basic hydrolysis yielded the isopalmitic acid (**10**). This acid was converted to the acid chloride (**11**) and then coupled to the sodium salt (*S*)-camphor sultam (**3**) in toluene according to the method of Oppolzer.[17] Face-selective alkylation was achieved using n-butyl lithium in THF:DMPU (4:1) and methyl iodide at –78 °C.[17] The diasteroselectivity of the alkylations was 95:5 as determined by ^{1}H NMR analysis[19] and a single recrytallisation gave diastereomerically pure **13**. The (*S*)-camphor sultam was removed by peroxide assisted LiOH hydrolysis[20] to give 2-(*R*)-14-dimethylpentadecanoic acid (**14**). This was coupled to L-glutamic acid dibenzyl ester yielding **15**, which was finally debenzylated by catalytic hydrogen transfer reduction using aqueous formic acid and Pd /C, to give (*R*)-L-(**2**).

As both (*S*) and (*R*)-camphor sultams are commercially available, as well as the (L)- and (D)-dibenzyl glutamic acids, this strategy enabled preparation of all four diastereomers of the *A. ludens* HMP (Scheme 2).

The (*R*)-L-(**2**) and (*S*)-D-(**2**) enantiomers had identical ^{1}H NMR spectral properties to the isolated natural product while the diastereomeric (*S*)-L-(**2**) and (*R*)-D-(**2**) enantiomers showed minor differences, particularly for the methyl doublet at C(2) and the NH signal. More importantly, the diastereomers could also be distinguished under specific HPLC conditions (Nucleosil-100-7um-Phenyl, 10 × 250 mm, 4 ml/min, 40/60% acetonitrile/water, 0.1% formic acid, 195 nm UV detection). The retention time for (*R*)-L-(**2**) and (*S*)-D-(**2**) under these conditions was 46 min. whereas (*S*)-L-(**2**) and (*R*)-D-(**2**) eluted after 48 min. The natural pheromone eluted as a single peak after 46 min, ruling out the (*S*)-L-(**2**) and (*R*)-D-(**2**) diastereomers as the natural product. Since the chirality of the 2-methyl group of the isopalmitic acid moiety could be confidently predicted from the face selective alkylation of **12** (Scheme 1) and the configuration of the amino acid was secure, we were confident at this stage that the natural product possessed the (*R*)-L configuration. With all the diasteromers of natural product in hand, biological activity would deliver the final confirmation. As the isolated natural product showed

Table 1. Biological activity of HMP compounds. Electrophysiology and behavioral laboratory tests (unless orthewise stated) were performed with *A. ludens* females. The field test was carried out with *A. obliqua* (see references [7] and [23] for details).

Entry	Compound	Biological Activity		
		Electro-physiology 1 ppm Spikes/ msec	Behavioral Laboratory Test 100 ppm DC	Test 100 ppm Efficacy % (Abbott)
1	Natural Product		78	90	----
2	(R)-L-(**2**)		76	82.3	----
3	(R)-D-(**2**)		50	23.2	----
4	(S)-L-(**2**)		16	----	----
5	(S)-D-(**2**)		6	----	----
6	L-(**17**)		25	23.7	----
7	D-(**17**)		2	----	----
8	(R)-L-(**2**)		72[a]	----	----
9	(R)-L-(**2**)		67[b]	----	----
10	(R)-L-(**22**)		78	84.8	64.37
11	(R)-D-(**22**)		23	----	----
12	(R)-L-(**29**)		----	84.3	----
13	(S)-L-(**29**)		----	61.7	----
14	(R/S)-L-(**29**) *Anastrephamide*		----	78.4	77.1

[a] Electrophysiology with *A. striata.* [b] Electrophysiology with *A. serpentina*

only one peak under the above-mentioned HPLC conditions, this also confirmed our suspicion that partial racemisation had occurred in the GC amino acid chirality determination, and that the isolated sample of natural product was in fact a single diastereomer.[21]

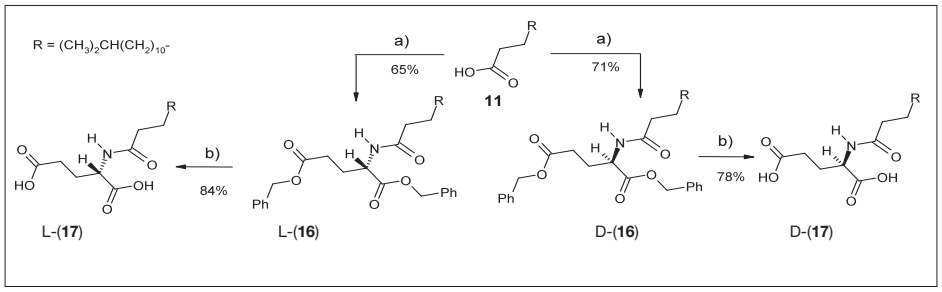
En route to the natural product, we were in a position to prepare compounds that would determine the importance of the 2-methyl group in isopalmitic moiety, simply by coupling the isopalmitic acid (**10**) to L- and D-dibenzyl glutamic acids, yielding L-(**16**) and D-(**16**), which were deprotected to yield the 2-desmethyl natural product analogues L-(**17**) and D-(**18**) (Scheme 3).

4. Biological Activity. Some Requirements for HMP Deterring Activity

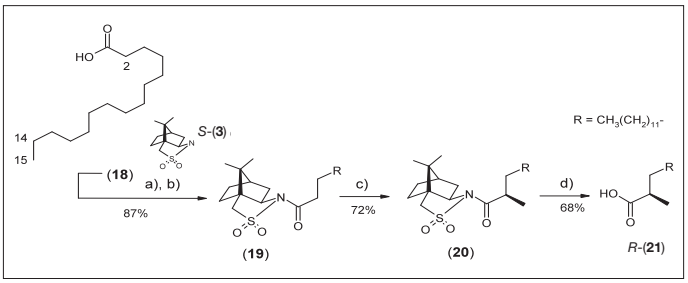
All of the diastereomers of the natural product (**2**), the corresponding dibenzyl esters (**15**), and 2-desmethyl natural products (**17**) described in Section 3 were now subjected to electrophysiology testing. The results for these evaluations are shown in Table 1.

These results clearly show that the synthetic (*R*)-L-(**2**) has the same activity as the natural product obtained from bioassay guided isolation from *A. ludens* faeces (entries 1 and 2), and that (*R*)-L-(**2**) had superior activity to the other diasteromers (compare entries 2 with 3, 4 and 5). This, combined with the identical spectroscopic and HPLC chromatographic properties of synthetic and natural *A. ludens* HMP, unambiguously confirms that the natural product has the *R* configuration at the 2-methyl position of the isopalmitic acid, and the L-amino acid configuration. Some other points can be extracted from Table 1: The L-amino acid configuration gives compounds of superior activity (compare entries 2 with 3, and 4 with 5). The 2-methyl group of the isopamitic acid moiety is required for activity (compare entry 2 with 6), and the preferred stereochemistry is the *R* configuration (compare entry 2 with 4). Although not depicted in Table 1, we also showed that esters of the glutamic acid moiety led to compounds (**15** and **16**) which showed no activity whatsoever in the electrophysiology tests.

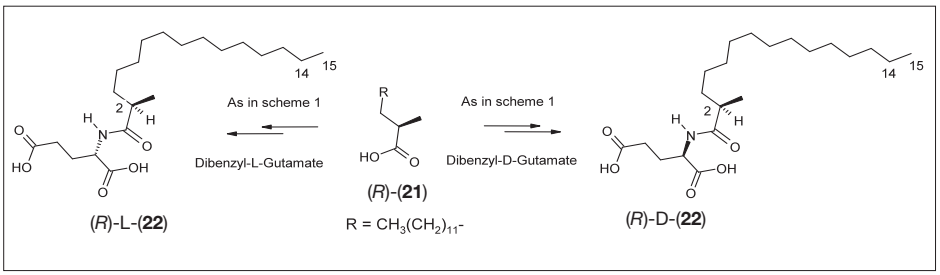
To confirm that the electrophysiology results translate to a HMP deterring effect, compounds (*R*)-L-(**2**), (*R*)-D-(**2**), and L-(**17**) were studied in the behavioural test with living *A. ludens*, as described in Section 1. As shown in Table 1, (*R*)-L-(**2**) was clearly more active than its (*R*)-D-(**2**) diastereomer, and the 2-desmethyl analogue L-(17). As expected (based on results with *A. ludens* faeces methanol extracts where complete interspecies cross recognition was observed between



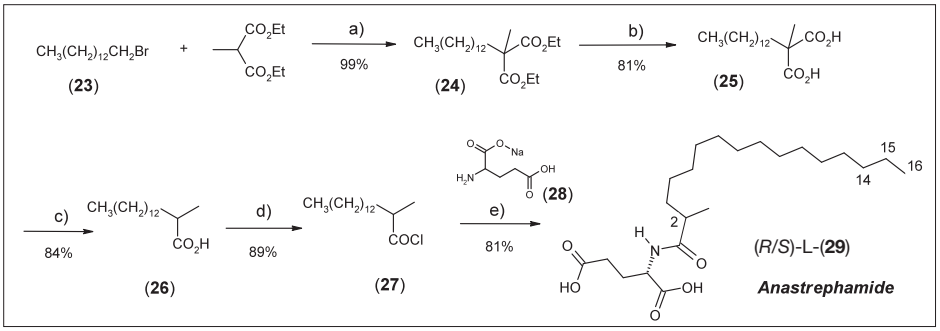
Scheme 3. Reagents and Conditions: a) L- or D-Glutamic acid dibenzyl ester 4-toluenesulfonate, EDC, Et_3N, cat. DMAP, CH_2Cl_2. b) HCO_2H (88% in H_2O):MeOH, (4:1), Pd/C (10%).



Scheme 4. Reagents and Conditions: a) $SOCl_2$, cat. DMF, r.t. b) NaH, toluene, r.t. c) n-BuLi, THF:DMPU (4:1), –78 °C, then add MeI –78 °C to r.t. d) LiOH, H_2O_2, THF:H_2O (9:1).



Scheme 5.



Scheme 6.

Anastrepha sp. in electrophysiology, behavioural, and field tests) the synthetic natural product (*R*)-L-(**2**) also showed complete interspecies cross recognition with *A. striata* and *A. serpentina* (Table 1, entries 8 and 9).

5. Simplification of the Natural Product

As we had shown that the methyl at C(2) of the isopalmitic acid was required for optimal activity, the next most obvious simplification, perhaps expected to have less impact upon biological activity, was the removal of methyl group at C(14) of the isopalmitic acid chain. The 14-desmethyl derivatives ((*R*)-L-(**22**) and (*R*)-D-(**22**)) were easily prepared by routes analogously to those described for the natural product from commercially available pentanedecanoic acid (Schemes 4 and 5).

The electrophysiology results for (*R*)-L-(**22**) confirmed that the natural product could be simplified, and that the (*R*)-L configuration was more active than the (*R*)-D configuration (Table 1, entries 10 and 11). In the behavioural test, the 14 desmethyl derivative (*R*)-L-(**22**) also showed excellent activity (Table 1, entry 10). At this stage, it will not go unnoticed to the reader, that the HMP of *Rhagoletis cerasi* and that of *Anastrepha ludens* have similarities in their structures, *i.e.* both containing a long fatty acid residue attached to an amino acid. In the case of **1**, the acid is a palmitic acid. As palmitic acid is a naturally occurring compound, we envisaged that a 2-methyl palmitic acid attached to L-glutamic acid might offer some benefits. Further, at this stage we knew that diastereomers differing in the configuration of the C(2) methyl of the fatty acid chain could be separated by HPLC. Thus, racemic 2-methyl palmitic acid (**26**) was prepared very simply according to the method shown in Scheme 6.[22] This was converted to the acid chloride (**27**), which could be directly coupled to L-glutamic acid monosodium salt (**28**) in THF using anhydrous LiCl to solubilise the mixture. This gave a 1:1 diastereomeric mixture of (*R/S*)-L-(**29**). The diastereomers could be separated by preparative HPLC using the conditions previously described for **2**. The first eluting diastereomer eluted after 53 min and the second after 56 min. These were assumed to be (*R*)-L-(**29**) and (*S*)-L-(**29**), respectively.[23] All three compounds were tested in the behavioural assay where it was shown that there was little difference in the activity between the distereomeric mixture, and the separated isomers (Table 1, entries 12, 13 and 14).

6. Field Testing

Based upon the results of the behavioural tests, and the clear advantages of ease of synthesis, we chose to prepare large amounts of (*R*)-L-(**22**), the 14-desmethyl derivative of *A. ludens* HMP, and (*R/S*)-L-(**29**), (which we have named *Anastrephamide*) to test in fruit orchards. Tests in tropical plum (*Spondias purpurea*) consisted of applying the compounds (100 ppm in water) with a knapsack sprayer to single fruit-bearing branches of *S. purpurea* trees visited by *Anastrepha obliqua* females. The results reported in Table 1[9] show that (*R*)-L-(**22**), reduced infestation in plums by 64% and *Anastrephamide* ((*R/S*)-L-(**22**)) by 77%, compared to untreated trees.

7. Conclusion

In conclusion, we have been able to isolate the HMP of *Anasterpha ludens*, drastically been able to simplify its structure whilst maintaining the biological activity against fruit infestation by *A. ludens* and other flies of this genus such as *A. obliqua*, *A. striata*, and *A. serpentina* under labora-

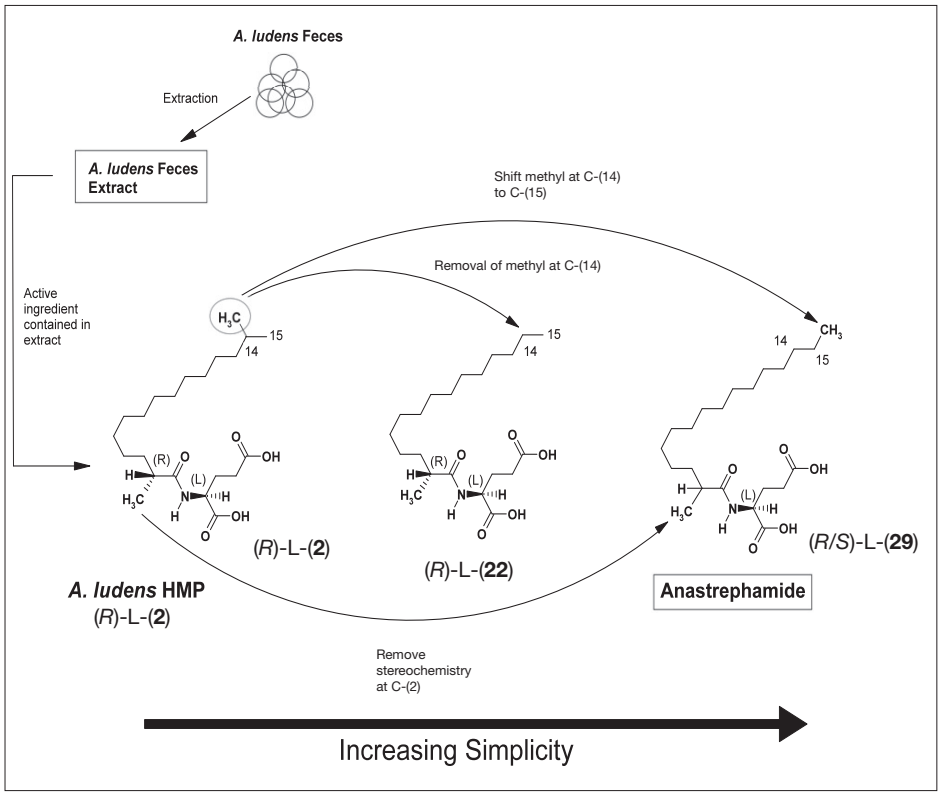


Fig. 4. Methanol extraction of *A. ludens* faeces leads to the *A. ludens* extract. The active principle contained in this extract is the host marking pheromone (HMP) of *A. ludens* HMP ((*R*)-L-(**2**)) which has the structure shown in the Figure. Leaving out the methyl group at C(14) greatly simplifies the synthesis of the second compound (*R*)-L-(**22**) used in this study. For the third compound discussed here (which we have named *Anastrephamide*), the methyl group was shifted to C(15), and the stereochemistry at C(2) was also discarded.

tory and field conditions. The work is summarised in Fig. 4.

Further studies will be required to ascertain if the deterrent effect of synthetic HMP derivatives will be successful in a crop protection scenario as such, or whether this 'push' strategy has to be combined with a 'pull' strategy were the females deterred from ovipositing are attracted to a poison trap as flies deterred from treated fruit and trees are not killed but fly away in search of clean, untreated fruit. Another option would be to treat certain rows with the synthetic HMP and treat a number of 'pull' rows with conventional insecticides to kill the flies moving away from the HMP-treated trees.

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