Colour-scent associations in a tropical orchid: Three colours but two odours

Roxane Delle-Vedove, Nicolas Julliet, Jean-Marie Bessière, Claude Grison, Nicolas Barthes, Thierry Pailler, Laurent Dormont, Bertrand Schatz.

Centre d’Ecologie Fonctionnelle et Evolutive (CEFE), UMR CNRS 5175, 1919 route de Mende, 34293 Montpellier cedex 5, France
UMR53, Laboratoire des Peuplements Végétaux et Bioagresseurs en Milieu Tropical, 15 Avenue René Cassin, BP 7151, 97415 St Denis de la Réunion cedex 9, France
Ecole Nationale Supérieure de Chimie de Montpellier, 8 rue de l’Ecole Normale, 34296 Montpellier cedex 5, France

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Colour and scent are the major pollinator attractants to flowers, and their production may be linked by shared biosynthetic pathways. Species with polymorphic floral traits are particularly relevant to study the joint evolution of floral traits. We used in this study the tropical orchid Calanthe sylvatica from Réunion Island. Three distinct colour varieties are observed, presenting lilac, white or purple flowers, and named respectively C. sylvatica var. lilacina (hereafter referred as var. lilacina), C. sylvatica var. alba (var. alba) and C. sylvatica var. purpurea (var. purpurea). We investigated the composition of the floral scent produced by these colour varieties using the non-invasive SPME technique in the wild. Scent emissions are dominated by aromatic compounds. Nevertheless, the presence of the terpenoid (E)-4,8-dimethyl-1,3,7-triene (DMNT) is diagnostic of var. purpurea, with the volatile organic compounds (VOC) produced by some individuals containing up to 60% of DMNT. We evidence specific colour-scent associations in C. sylvatica, with two distinct scent profiles in the three colour varieties: the lilacina-like profile containing no or very little DMNT (<2%) and the purpurea-like profile containing DMNT (>2%). Calanthe sylvatica var. alba individuals group with one or the other scent profile independently of their population of origin. We suggest that white-flowered individuals have evolved at least twice, once from var. lilacina and at least once from var. purpurea after the colonisation of la Réunion. White-flowered individuals may have been favoured by the particular pollinator fauna characterising the island. These flowering varieties of C. sylvatica, which display three colours but two scents profiles prove that colour is not always a good indicator of odour and that colour-scent associations may be complex, depending on pollination ecology of the populations concerned.

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Introduction

Polymorphism in floral traits is widespread in animal-pollinated plants, and ecologists have been long intrigued by its emergence and maintenance (Weiss, 1995). Except for the case of specific and obligate plant–pollinator associations, where low intraspecific variation of floral traits has been found (Dufaÿ et al., 2004; Hossaert-McKey et al., 2010; Svensson et al., 2006), high polymorphism of pollinator-attractant floral traits has been documented at individual or population levels in numbers of flowering plants. Variation occurs for different floral traits such as colour (Dormont et al., 2010; Gigord et al., 2001; Waser and Price, 1981; Weiss, 1995), odour (Azuma et al., 2001; Knudsen et al., 2006; Majetic et al., 2009; Salzmann et al., 2007; Tollsten and Bergström, 1993), flower size (Dickson and Petit, 2006) and flowering phenology (Parra-Tabla and Vargas, 2007). These floral traits are the principal detectable and attractive signals for pollinators and are used by animal-pollinated plants generally in addition to a reward, which ensures their repeated visits. Investigations in plant reproduction have long asserted that non-random combinations of flower colour, shape and fragrance have evolved in response to directional selection exerted by specific classes of effective animal pollinators (Faegri and van der Pijl, 1979; Fenster et al., 2004; Johnson et al., 1997; Raguso et al., 2003). Pollinators show preferences for specific odour, colour or shape according to innate preferences but also influenced by their considerable learning capabilities and sensory flexibility (Chittka et al., 1999; Daly and Smith, 2000; Goyret et al., 2008; Kelber, 2010). Such associative learning is based on floral characters or floral rewards of different kinds (nectar, pollen, oils, etc.) or on associations of these traits (Giurfa et al., 1997; Raine and Chittka, 2007). Learning in insects is complex and depend on discrimination capacities of the insect. These are first conditioned by the species specific sensory machinery of insects and, environmental conditions as illumination or the presence of contrasting floral species (Dyer and Chittka, 2004;...
Kelber, 2010). Discrimination capacities could also be modulated by previous experiences and the combined use of visual, gustatory or olfactory inputs (Avarguè-Weber et al., 2010; Giurfa et al., 1994; Kelber, 2010). These capacities are the basis of “pollinator constancy”, in which a pollinator visiting one flowering plant is more likely to move to another plant of the same morph than would be expected based on plant frequency in the population (Jones and Reithel, 2001; Waser, 1986). This behaviour is expected to exert stabilizing selection, leading to uniformity of attractive floral traits (Raguso et al., 2003). For example, studies of pollination syndromes give rise to the hypothesis that specialization of flowers on pollination by nocturnal hawkmoth leads to the production of white-flowered morphs, which emit more aromatic (i.e. benzoid compounds) compounds (especially alcohols and esters: Majetic et al., 2007; Raguso et al., 2003). Recently, the generality of floral syndromes has been questioned based on the apparent commonness of generalized pollination systems, although highly specialized pollination systems are also well documented (Cozzolino et al., 2005; Johnson and Steiner, 2000). A second concern about pollination syndromes is that evolution of floral traits is not solely the result of selection by pollinators (or other agents) but may also reflect phylogenetic constraints (Knudsen et al., 2006; Raguso et al., 2003).

Thus, floral trait polymorphism at individual or population levels has been documented in a large number of flowering plants. Current explanations of the maintenance of variable floral traits in flowering plants traditionally rely on the key role of insects through pollinator-mediated selection. Polymorphism may be maintained by the existence of several effective pollinator species with different foraging preferences. For example, hawkmoth and hummingbird pollinators impose divergent selection respectively on yellow and red morphs of Mimulus aurantiacus (Scrophulariaceae) (Streisfeld and Kohn, 2007). Pollinators may also exert temporally or spatially fluctuating selection pressures, when their abundance and assemblage structure are variable (Brown and Clegg, 1984; Eckhart et al., 2006; Waser and Price, 1981). More recently, it has been proposed that floral trait variation may also be maintained by multiple and conflicting selection pressures, both by biotic agents such as pollinators and herbivores and by abiotic factors (such as heat or drought). For example in Ipomoea purpurea (Convolvulaceae), pigment compounds serve to attract pollinators to the flowers and also increase tolerance of water and heat stress in vegetative parts (Warren and Mackenzie, 2001).

Colour and scent are generally the main stimuli that attract insects to flowers (Chittka and Raine, 2006; Dobson, 1994) and in some cases, the combined effect of colour and odour allow the pollinators to distinguish between different flowering plants better than if the two signals are presented separately (Galizia et al., 2004; Kunze and Gumbert, 2001). Depending on the species, a combination of both sensory cues can be used. Giurfa et al. (1994) demonstrated that honeybees could supplement visual cues by using olfactory inputs to enhance their foraging activity. Alternatively, male solitary bees used sequentially olfactory signals from a distance and then visual cues to guide them towards Ophrys heldreichii (Streinzer et al., 2009). Recently, authors have highlighted the existence of variation in scent emissions of different colour morphs of the same flowering species (Li et al., 2006; Majetic et al., 2007; Odell et al., 1999; Salzmann and Schiestl, 2007). Colour-scent relationships may be complex and often rely on shared biosynthetic pathways (Armbruster, 2002; Majetic et al., 2007), implying that any mutation in a gene coding for an enzyme or a regulatory element will have an impact both on colour and scent emitted. Zucker et al. (2002) showed for example that the suppression of a single key enzyme led to the formation of white-flowered mutants with higher amounts of aromatic compounds (methyl benzoate) than in coloured individuals. This suggests that specific flower colour-scent associations could be a consequence of conserved biochemical pathways (Armbruster, 2002) and may not be dissociated—or formed—by natural selection (Majetic et al., 2007).

Species polymorphic for floral traits are ideal model systems to study the joint evolution of floral traits and their impact on plant reproduction. In this context, orchids are highly pertinent models for studying plant–pollinator interactions as they present a great variety of floral traits and trait associations. This is mirrored by the great diversity of reproductive strategies in orchids. One of the most intriguing of these strategies is deceptive pollination (i.e. nectarless flowers), which concerns about one third of orchid species. Several strategies of pollinator attraction are identified in these species (see Jersakova et al. (2006) for a review). Deceptive pollination often rely on newly emerged naive insects or may benefit from facilitation effect by the surrounding rewarding species community (Johnson et al., 2003; Juillet et al., 2007). Several species of deceptive orchids display floral trait polymorphism supposed to be advantageous to avoid associative learning potentially performed by pollinators (Nilsson, 1992; Jersakova et al., 2006). In orchids, as in other insect-pollinated plants, the extent to which colour and odour polymorphism may influence reproductive success remains poorly understood (Dormont et al., 2010). We investigated colour-scent associations in the floral-polymorphic orchid Calanthe sylvatica (Orchidaceae) in Réunion Island presenting three different colour varieties. A first study revealed that the three different colour varieties occur in spatially separated populations, flower at different periods of the year and also present differences in flower morphology, for example in spur length (Juillet et al., 2010). They also achieve different fruit set, which is higher in white varieties than in coloured ones. Here we analysed the scent production of C. sylvatica and attempted to determine whether there are associations between floral colour and scent in this species. We address three questions:

1. What is the scent composition of C. sylvatica flowers?
2. Does floral scent vary among individuals within colour varieties of C. sylvatica?
3. Are there specific colour-scent associations among colour varieties?

2. Results and discussion

2.1. Scent characterization of C. sylvatica

The scent of C. sylvatica individuals was composed of 24 identified compounds, all of which were present in individuals of all of the three colour categories but in different relative proportions (Table 1). These compounds were either terpenoids or aromatic compounds. The scent of C. sylvatica flowers is dominated, in most individuals, by aromatic compounds that are relatively frequent in floral scents (Knudsen et al., 2006) and often cited as typical attractants for moth pollinators (Hubert et al., 2005; Majetic et al., 2007; Raguso et al., 2003). Three aromatic compounds accounted for a large fraction (55–75%) of the scent profiles of C. sylvatica (Table 1): methyl benzoate, benzyl alcohol and benzyl benzoate. Four other compounds, namely (E)-4,8-dimethylnona-1,3,7-triene (DMNT), (E)-dendrolasine, benzyl cyanide and methyl salicylate were also relatively important in the odour bouquet (each more than 5% in at least one colour variety), and dominated the odour emission of some individuals (e.g. up to 62% of DMNT in some var. purpurea individuals, Table 1). Fifteen compounds presented significant differences in abundance among colour varieties (Table 1). Of these, 13 presented heterogeneous values in relative abundance for var. alba individuals. Overall, a principal component analysis (PCA) performed on the relative proportions of all
compounds revealed that the two first axes explained 78.54% of the total variance (Fig. 1a). Axis 1 was mainly influenced by the relative amount of two compounds: methyl benzoate and DMNT (Fig. 1b) from which DMNT was the sole to present significant differences among varieties (Table 1). Most individuals were clustered into two groups: one cluster contained all individuals of var. lilacina and some of the var. alba individuals and the second cluster contained almost all var. purpurea individuals and most of the remaining var. alba individuals.

Dendrolasine and benzyl cyanide are not common in flower scents, each having been reported only once in floral scent, the first (Arditti, 1998), and are thus potentially biologically relevant in the pollination of C. sylvestris. Further GC–EAD analysis will allow us in future studies to discriminate the biological role of scent compounds in the C. sylvestris system. These results are consistent with what we know or suppose on C. sylvestris pollinators. The pollinator fauna of Réunion Island is characterized by a poorly diversified insect fauna, except in nocturnal Lepidoptera, and it features a particular high diversity of Sphingidae species (Martiré and Rochat, 2008). The diurnal hawkmoth Macroglossum stellatarum was observed visiting C. sylvestris var. lilacina (T. Pailler, personal observation). Complementary field observations are necessary to extend our knowledge of the pollinators of C. sylvestris.

### 2.2. Colour-scent associations in C. sylvestris and the potential impact of biochemical constraints

#### 2.2.1. Between lilac- and purple-coloured varieties

Despite the large amount of aromatic compounds emitted by all varieties of C. sylvestris, scent differentiation between the lilac and purple varieties is mostly accounted for by their terpenoid composition, especially regarding the amount of DMNT: individuals of var. purpurea produce on average large amounts of DMNT (mean ± SE = 20.42 ± 4.1% and var. lilacina individuals always produce low (<1.5%) quantities of it (0.13 ± 0.06%). Purple pigments are frequently anthocyanins (Clegg and Durbin, 2000), which are present in the genus Calanthe (Arditti, 1992). The result here found seem to be contradictory to the “biochemical pathway” hypothesis, as flavonoid pigments, especially anthocyanins, are produced by the aromatic pathways (Zucker et al., 2002) and are thus not directly related to production of terpenoids. Salzmann et al. (2007) also found an association of the red carotenoid pigments, which are linked to the terpenoid pathway (Dudareva et al., 2004), if carotenoids are
responsible of coloration in Calanthe, production of greater proportions of terpenoid scent compounds could simply be a byproduct of being red. In the absence of a complete study of pigments involved in lilac and red colorations, the hypothesis that biochemical constraints lead to associations between colour and scent cannot be firmly rejected.

2.2.2. Between coloured and white varieties

The PCA reveal that individuals clustered in two groups, the first composed by all individuals of var. lilacina and some of the var. alba individuals and the second by almost all var. purpurea individuals and most of the remaining var. alba individuals. Some var. alba individuals and one var. purpurea individual were classified as “intermediate”. A first discriminant function analysis (DFA) on the relative proportion of scent compounds allowed separation of the three colour varieties \( F = 201.78, \text{ df} = 48, p = 3.76 \times 10^{-9} \), but not completely, especially for var. alba individuals. In fact, without prior knowledge of the colour variety, 5 of the 18 var. alba individuals were misclassified in coloured varieties. We then tested another hypothesis of assignation. Given that the relative proportion of DMNT allows discriminating among colour varieties (Table 1, Fig. 2a), we recoded var. alba individuals according to the presence or absence of DMNT. This allowed us to test whether var. alba individuals are well assigned into the lilacina or purpurea-like scent profiles. This new classification was well supported by a second DFA \( F = 296.40, \text{ df} = 24, p = 4.32 \times 10^{-8} \), with all
individuals being well classified in one or the other scent-profile. The ANOVA on the individual contributions to first axis was thus highly significant ($F_{1,58} = 21.76, \ p = 1.96 \times 10^{-5}$). This detailed study of var. alba individuals revealed that they fall for one part in a lilacina-like profile, and for the other part in a purpurea-like profile, as shown by the DFA and the PCA (Figs. 1a and 2b) and well exemplified by amounts of the terpenoid DMNT in Fig. 2a.

If biochemical constraints are important in colour-scent associations, we would expect to find consistent differences between coloured and white-flowered varieties, and particularly an increased production of aromatic compounds in the latter (Majetic et al., 2008; Zucker et al., 2002). Surprisingly, white individuals in C. sylvatica did not show such a pattern (Table 1). Our results clearly diverged from expectations of the biochemical-constraint hypothesis as no differentiation was found between coloured and white-flowered varieties of C. sylvatica.

2.2.3. Within the white variety

Another intriguing result of the study is the scent differentiation between two groups of white individuals. We present the PCA figure with the new grouping in two scent profiles in Fig. 2b. Var. alba individuals presenting lilacina-like scent (without DMNT) and purpurea-like scent (with DMNT) were equally represented in our sample ($n = 10$ and $n = 8$, respectively), and were present in all three sampled populations.

Studies of scent variations between colour morphs have identified variations in white flower scent among populations (Dormont et al., 2010; Majetic et al., 2007). In C. sylvatica, two scent profiles can be found in a single white-flowered population and they matched the scent profiles of one or the other coloured variety. Selection by abiotic or biotic factors appears more likely to have shaped colour-scent associations in C. sylvatica than biochemical constraints. This situation, in which pollinators encounter a white-flowered morph displaying two kinds of odours, is to the best of our knowledge unique in orchids. It allows us to raise new exciting questions. In the following, we discuss the origin and maintenance of these two profiles in white-flowered C. sylvatica.

2.3. Evolution of colour-scent associations in C. sylvatica

2.3.1. Differentiation between varieties lilacina and purpurea

How did these colour and scent variations arise in Réunion Island? C. sylvatica probably colonized the Island from East Africa or Madagascar. Although data on herbarium specimens from these countries (East African Herbarium, Nairobi, Kenya; Herbarium of the National Museum of Natural History, Paris, France) mention various flower colours, it is unknown whether the three colour varieties colonized the island independently or have evolved locally. Orchids usually do not set fruit in the absence of animal pollinators, and fruit set is often low, particularly for nectarless tropical species (Tremblay et al., 2005). C. sylvatica is no exception to this rule (5.17 ± 9.34% of open flower setting fruit; Juillet et al., 2010), and there is great potential for pollinator agents to select for specific floral traits combinations attractive for them. For nectarless species, the around rewarding floral diversity could shape pollinator preferences and could also lead to mimicry (Johnson et al., 2003). For example, var. lilacina could take advantage of the pollinators attracted by the reward shrub Chassalia coriloides, which often bloom in the same populations and present flowers of the same colour (Juillet et al., 2010). Alternatively, the colour-scent associations documented here could also be independent from each other, resulting from divergent environmental conditions or from selective pressures on each trait exerted by forces unrelated to pollinators. Especially, we know that DMNT is often associated with indirect plant defence (Boland et al., 1992) and, even if we did not see any special damages on var. purpurea individuals in the field, DMNT could be produced in response of biotic pathogens pressure.

2.3.2. Polymorphism within var. alba

Considering the two scent profiles found in var. alba individuals, we propose that these groups may be derived from two independent mutations, once in var. lilacina and once in var. purpurea individuals, after the colonisation of Réunion Island. Loss-of-function mutations are often the cause of transitions from coloured to white flowers, and are more common than the reverse transitions (review in Rausher (2008)). Such mutations in colour-coding genes, or regulatory elements affecting them, may have occurred in C. sylvatica. However, the exact scenario for the evolution of var. purpurea and var. lilacina remains unknown. A detailed genetic study would enable us to test specifically this hypothesis.

As both scent profiles co-occur within white-flowered populations, this would imply in turn that mutant white phenotypes have converged in adapting to conditions ecologically different than those to which the parental coloured varieties are adapted, occurring at lower altitude and flowering later (Juillet et al., 2010). This convergence may have been driven by pollinator preferences and the facilitation effect of a guild of nectar-producing white co-flowering species, which represent a large part of the Réunion orchid flora (Jacquemyn et al., 2005, 2007). This convergence might also have been facilitated by the probable continuity between var. alba habitat (lower altitude) and habitat of the coloured varieties (mid to high altitude) before human colonisation (XVIIIth century) and the ensuing forest fragmentation (Strasberg et al., 2005).

Var. alba individuals from the two scent profiles were found in all three sampled populations. Some studies, mainly focused on flower colour, have hypothesized that this type of dimorphism should be adaptive for nectarless species, as it reduces the speed with which pollinators learn to avoid rewardless flowers, and generates negative frequency-dependent selection that could maintain the floral colour dimorphism (Gigord et al., 2001; Smithson and MacNair, 1997). To date, this form of selection has never been demonstrated to maintain different scent variants within populations. A complete study at the population level, quantifying the reproductive success of var. alba individuals according to their scent profiles and the frequency of these profiles, would enable evaluation of this hypothesis. Alternatively, pollinator observation should enable us to know if the different scent profile attract different species of them or elicit different pollinator's behaviour, leading to the maintenance of these polymorphisms.

3. Concluding remarks

We demonstrated that white individuals displayed two kinds of odours, and that the odours emitted by white individuals correspond to the odours emitted by each of the two coloured varieties. The consequence is that what the insect smells does not match what it sees. Floral polymorphisms are complex and strongly influenced not only by the pollinators, but also by several other selective agents such as herbivores and abiotic factors or even by pleiotropic effects of selection acting on other traits (Dormont et al., 2010; Rausher, 2008; Schaefer et al., 2004; Schaefer and Ruxton, 2009). Our study demonstrates an original case of colour-scent association and the importance of taking all floral traits into account when studying floral polymorphism (Raguso, 2008). The floral odour-colour combination has been treated in recent reviews as one of the main future issues in pollination ecology (Raguso, 2008; Rausher, 2008; Schaefer and Ruxton, 2009). The case of C. sylvatica suggests several ways to explore the ecological
consequences of discordance between visual and olfactory signals produced by floral traits.

4. Experimental

4.1. Study site and species

The study was conducted on Réunion Island, a volcanic island situated in the western Indian Ocean. Réunion Island is characterized by a steep altitudinal gradient ranging from 0 to 3070 m and a tropical climate. Mean annual rainfall ranges from 1500 mm on the east coast, to more than 8000 mm in high altitude. The island presents a highly diverse orchid flora, with a total of 135 species of which 42% are terrestrial (Jacquemyn et al., 2007).

Calanthe sylvatica (Thouars) Lindl. is a terrestrial orchid species with large and showy inflorescences and a pollination system relying on deception (absence of floral rewards) (Jacquemyn et al., 2007; Juillet et al., 2010). The species is colour-polymorphic (De Cordemoy, 1895) with three known varieties differing in colour, morphology, flowering phenology and altitudinal distribution (Juillet et al., 2010). Var. alba individuals grow at low altitude (0–1100 m), have white inflorescences and their flowering peaks in the late rainy season (March); var. lilacina and purpurea grow at higher altitudes (from 1100 m up to 1600 m) and bear respectively lilac and purple/red flowers. Var. lilacina flowers mainly in December and var. purpurea from December to May. The three colour varieties seldom co-occur in the same location.

Preliminary observations in natural conditions and knowledge of Réunion Island’s pollinator fauna suggest that this species might be pollinated by the diurnal hawkmoth M. milvus Boisduval (Sphingidae).

4.2. Odour sampling

For each colour variety, a total of three populations were randomly selected and VOC were extracted using solid-phase micro-extraction (SPME) technique, a non-destructive solvent-free sampling technique. The sampling of odour was carried out in situ on 58 individuals (6–10 individuals per population) from nine populations (three populations per colour variety), between 09h00 and 12h00.

Sampling by SPME was performed using 65 µm polydimethylsiloxane/divinylbenzene (PDMS–DVB) fibres (Supelco®, Sigma–Aldrich, Bellefonte, PA, USA). For each sampled individual, we enclosed the whole inflorescence in a polyethylene terephthalate bag (Nalophan®, Kalle Nalo GmbH, Wurstshüllen, Germany). An SPME fibre was then introduced with a manual holder into the bag and was adjusted in close proximity to flowers (1–2 cm) and left for VOC collection for 2 h. This duration was found in preliminary tests to be the best collection time. A sample of surrounding air was collected in the same way as a control. After sampling, fibres were brought back to the laboratory in a cool box, and stored at −20 °C until analysis.

4.3. Gas chromatography and mass spectrometry of VOCs

Gas chromatography and mass spectrometry (GC–MS) analyses of the SPME extracts were performed using electronic impact ionization mode on a Varian Saturn 2000 ion trap spectrometer, interfaced with a Varian CP-3800 apparatus. The Varian CP-3800 was equipped with a 1079 split-splitless injector (260°C) and a 30 m × 0.25 mm × 0.25 µm film thickness ID WCOT CPSil-8CB fused silica capillary column (Chrompack®, Bergen op Zoom, The Netherlands), with helium as carrier gas (1 ml/min), and programmed 2 min isothermal at 50°C, then increasing from 50°C to 220°C at 4°C/min. These analyses were performed at the “Plate-forme d’Analyses Chimiques en Ecologie” (Platform for Chemical Analyses in Ecology) of “IFR 119 Montpellier Environnement Biodiversité”. Mass spectra were recorded in electronic impact (EI) at 70 eV, and identified by comparison with data of the NIST 98 software library (Varian, Palo Alto, CA, USA). In addition, identification of some compounds has been confirmed by RI and Mass Spectrum comparison of the samples with commercial known standards GC–MS runs. For (E)-DMNT and (Z)-DMNT, an original ecological synthesis has been performed to confirm identification and isomerism since no commercial reference exist (see Section 4.4). Comparison of the chromatographs of the orchid samples with that of the control sample from the same population allowed removing the peaks corresponding either to adsorbent, to degradation of Nalophan®, or to volatiles present in the air surrounding the bags. Peaks were quantified using the Star Chromatography Software® and the proportion of each compound was expressed as the peak area relative to the total peak area for all compounds.

4.4. Revisited synthesis of DMNT

Despite its recurring interest in chemical ecology, DMNT has received no recent attention by chemists. Only few DMNT syntheses have been reported and involved Wittig reaction (Maurer et al., 1986). This reaction, although being useful so far in classical organic chemistry, requires rigorous experimental conditions, using of hazardous solvents and reagents, and it does not maximize atom economical. Indeed, it takes place at low temperature (−78 °C), under anhydrous conditions because of, among others, strong organic base reactant (LDA) and uses large amounts of solvents (THF and petroleum ether), both for the reaction and for the numerous treatments and purifications.

Recent cleaner method like metathesis, involving last generation of Grubbs’ catalysts, is getting more and more interest in the field of pheromone synthesis (Mori and Tashiro, 2004) but is not suitable for DMNT preparation as it would engage 1,3-butadiene and leads to polymerisation products.

We herein report an innovative access to DMNT incorporating a green chemistry approach: solvent free and highly concentrated reactions. The synthetic strategy includes the sequence aqueous Wittig-Horner-Emmons reaction/saponification (Courot et al., 1992; Villieras and Rambaud, 1982; Villieras et al., 1985), followed by a copper-catalysed decarboxylation (Fig. 3) (Gooßen et al., 2007).

Synthetic sample has been analysed by 1H NMR (Bruker NMR Advance 300 MHz – CDCl3) δ (ppm): 1.70 (s, 3H, Me), 1.78 (s, 3H, Me), 1.85 (s, 1.89H Me, δ enantiomer 63%), 1.87 (s, 1.11H Me, Z enantiomer 37%), 2.13–2.30 (m, 4H, CH2), 5.01–5.22 (m, 3H, CH2 vinylogous and CH), 5.95 (broad d, 1H, δH-H = 12 Hz, CH), 6.65 (t, 1H, δH-H = 18 Hz and δH-H = 12 Hz, CH).

DMNT has a Rf of 0.8 in pure hexane (0.3 for citral) on TLC (Merck – 5355 – Kieselgel 60-F254), detection being carried out by UV or by iodine.

The diastereomer ratio was easily determined from the integration of the protons signals (of the methyl carried by C-4) in the 1H NMR data (δH-δH = 1.85 ppm and δH-δH = 1.87 ppm). (E)-DMNT has been identified as major compound (62%). The obtained values clearly demonstrated the configurational integrity of the C=C double bond in the successive reactions used to obtain DMNT.

This sample analysed by GC/MS (see Section 4.3, for analysing conditions) showed retention indexes of 1110 for (E)-DMNT (63%) and 1089 for (Z)-DMNT (37%) which permits the chromatographic assignments in our study.

4.5. Data analysis

The scent composition of each C. sylvatica colour variety was compared using F tests followed by Tukey–Kramer multiple com-


Comparison tests, for each compound independently. The relative scent composition among colour varieties was tested with a principal component analysis (PCA) and a discriminant function analysis (DFA) on the matrix of relative proportion of each compound. All analyses were done using R 2.8.0 (R Development Core Team, 2008).

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References


Maurer, B., Hauser, A., Froidevaux, J.C., 1986. (E)-4,8-dimethyl-1,3,7-nonatriene and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, two unusual hydrocarbons from Rhinospermum heterogene VI. Selectivite de la reaction sur des composes bifonctionnels. Synthesis 11, 924.


