NMR-Based Metabolic Profiling of *Anigozanthos* Floral Nectar

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Nuclear magnetic resonance spectroscopic methods have been used to characterize the chemical composition of floral nectar of *Anigozanthos* species with a minimum of sample preparation and without derivatization. The nectar of this passerine-pollinated plant is largely dominated by glucose and fructose, while sucrose occurs only at a minor level. Tyrosine, several additional amino acids, and a variety of carboxylic acids were identified and their concentrations estimated. A linear diarylheptanoid was detected as a trace component, marking the first time this type of secondary product in Hemodoraceae has been found.

Floral nectar composition, especially the relative proportion of the sugars and amino acids, plays an important role in plant−pollinator interaction and evolution. Our interest in the phytochemistry, biochemistry, and ecological interactions of plants of the Hemodoraceae family prompted us to study the metabolic profile of nectar of *Anigozanthos* species (subfamily Conostylideae). *Anigozanthos* flowers are reported to be pollinated by passerines, especially of the honeyeater family (Meliphagidae) and marsupials such as the honey possum (*Tarsipes rostratum*), which are the only nonflying mammals feeding exclusively on a diet of nectar and pollen.[1–3]

The septal nectaries of the *Anigozanthos* flowers are large compared to those of other Hemodoraceae.[4] Large nectaries seem to be an evolutionary adaptation: they allow nectar production to be increased, thus attracting bird pollinators.[5] The enlarged capacity for the production of nectar requires storage space accessible to the pollinators, which is provided by the special floral morphology of *Anigozanthos* flowers (Figure 1). The known pollinators and the availability of intensely blooming greenhouse *Anigozanthos* plants, which produce a relatively large quantity of nectar, facilitated the study of the nectar's metabolic profile.

Chemical analysis of nectar samples has been focused on the concentration and relative proportion of carbohydrates.[6–9] Secondary metabolites, which usually occur at low levels but are clearly involved in ecological interactions, have also been reported.[10] Depending on the target compounds, different methods were used for nectar analysis including various chromatographic and spectroscopic techniques, for example GC-MS. Due to its intrinsically low sensitivity, NMR spectroscopy has rarely been used to determine nectar composition, although it is a universal method for analyzing natural products containing protons and carbons, which are the most frequent atoms of nearly all biomolecules. Progress in NMR technology, especially the enhanced sensitivity of cryogenically cooled probes, has made it possible to analyze not only major sugars but also minor and trace nectar components qualitatively and quantitatively with a minimum of sample preparation and without derivatization. Using *Anigozanthos flavidus* floral nectar as an ecologically relevant sample, we describe an NMR-based procedure for nectar analysis and report on a detailed metabolic profile. NMR spectroscopy was employed in three analytical steps: (1) ^{13}C NMR of untreated nectar, (2) ^{1}H NMR of untreated nectar, and (3) ^{1}H-detected NMR of nectar fractions enriched by solid-phase extraction (SPE). Using these methods, differently abundant nectar components, i.e., the major carbohydrates glucose and fructose (step 1), low-abundant compounds (step 2), and trace components (step 3), differing in their concentration by several orders of magnitude, were identified and quantitatively determined. Identification of the first linear diarylheptanoid in Hemodoraceae is also reported, and its biosynthetic implications are discussed.

Figure 1. Flower of *Anigozanthos flavidus,* from which nectar was collected (bar = 40 mm).

Results and Discussion

Major Carbohydrates of *Anigozanthos* Nectar. The analysis of many floral nectar samples has shown that carbohydrates are always the major constituents and that glucose, fructose, and sucrose are the dominant sugars.[8] However, the ratio of these three carbohydrates varies considerably in floral nectars of different plants and has been used to classify them.[11] Intraspecific and intraplant variations in volume and sugar composition of floral nectar have been shown to be less pronounced in greenhouse-grown plants.[12,13] Both total sugar content and relative proportion of sugar components have been correlated with pollinator type[8,14,15] but are still a matter of debate.[16]

Various methods such as paper chromatography,[15] colorimetry,[17] fluorimetry,[18] spectrophotometry,[19] HPLC,[20] HPLC combined with enzymatic and colorimetric techniques,[21] infrared spectroscopy,[22] and gas chromatographic methods[23] have been used to analyze total sugar content or individual carbohydrate components in nectars and other plant samples. Although suitable for the simultaneous determination of glucose, fructose, and sucrose in plant samples,[24] NMR spectroscopy, surprisingly, has not been a standard method of analyzing nectar composition.

In principle both ^{1}H and ^{13}C NMR techniques are useful for detecting sucrose and the two hexoses and discriminating among

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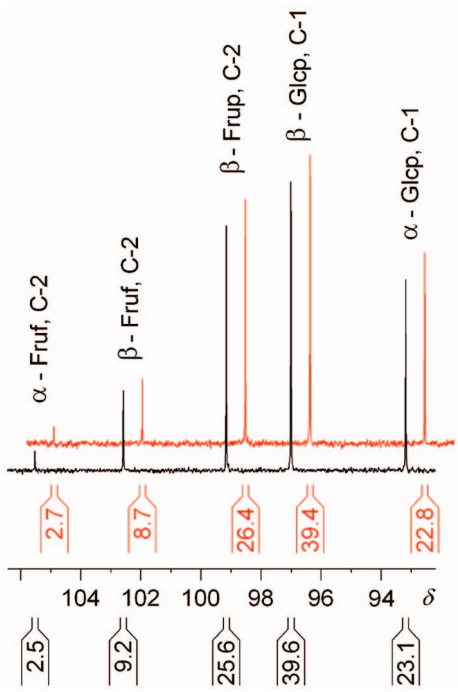

Figure 2. Partial ^{13}C NMR spectra (125 MHz) showing signals used to determine the proportion of fructose to glucose. Spectra were measured and processed under identical conditions (power gated decoupling; 30° flip angle; relaxation delay = 2 s, line broadening factor = 1) in K-Pi buffer (D_2O, pH 7.5). Black spectrum: nectar of *Anigozanthos flavidus* (50 μL) in 450 μL of buffer; red spectrum: mixture of fructose and glucose (1.0:1.2) in 50 μL of H_2O + 450 μL of buffer. Integrals are shown below (nectar) and above the axis (fructose + glucose mixture).

the various isomeric forms of fructose and glucose. Although much more sensitive, ^1H NMR suffers from a low dispersion of signals, resulting in the crowded spectral part between δ 4.1 and 3.2 (Figure 3), and, in H_2O/D_2O, interference of the doublets of H-$1_{\alpha\text{-glc}}$ (δ 5.21) and H-$1_{\beta\text{-glc}}$ (δ 4.62) with the large water signal between. The latter can be suppressed by presaturation or more efficiently by pulse sequences such as PURGE (presaturation utilizing relaxation gradients and echoes).[25] However, the residual HDO signal may still affect integral values of doublets of the β-protons at the anomeric centers of carbohydrates or obscure signals of minor components. In this study, we preferred ^{13}C NMR in cases where the nectar to be analyzed is available in sufficient amounts but recommend ^1H NMR for analyzing sugars in mass-limited nectar samples. Thus, in our study on *Anigozanthos* nectar, we chose the less sensitive ^{13}C NMR technique to analyze the two major carbohydrates, glucose and fructose, because of higher signal dispersion and independence from using water suppression. Power gated decoupling was used because it takes advantage of sensitivity enhancement through the nuclear Overhauser effect.

The floral nectar of *A. flavidus* was used in this study. In addition, nectar samples of *A. manglesii* and *A. humilis* were used in some experiments. For carbohydrate analysis, nectar was collected from the flowers of greenhouse-grown *Anigozanthos* plants and subjected to ^{13}C NMR analysis (in deuterated K-Pi buffer pH 7.5) immediately after harvest or after storage at −20 °C. Using a TCI cryoprobe, we found that 50 μL of nectar was sufficient to acquire NMR spectra of an S/N of ~70 (for the largest signal, C-1 of β-D-glucopyranose) accumulated from 1k FIDs within approximately 1 h. However, 4k FIDs were accumulated to more accurately integrate all signals used for quantification, including the small resonance of C-2 of α-fruf at δ 105.5, resulting in a S/N of ~140 for C-1 of β-glcp. Standards of glucose and fructose were measured under identical conditions in order to confirm reported ^{13}C NMR signal assignment.[26]

The power gated decoupled ^{13}C spectra displayed five sets of signals, two of glucose isomers α-D-glucopyranose (α-glcp) and β-D-glucopyranose (β-glcp) and three of fructose isomers α-D-fructofuranose (α-fruf), β-D-fructofuranose (β-fruf), and β-D-fructopyranose (β-frup). Acyclic keto or aldehyde forms were not detected due to their low levels, which are <1% of the total amount of glucose or fructose, respectively. Interestingly, sucrose was also not detected by ^{13}C NMR, indicating that this sugar does not occur in significant levels in floral nectar samples of the three *Anigozanthos* species. The signals of the anomeric carbon atoms of the two glucose and three fructose isomers, which appear in the spectrum as well-separated resonance lines at δ 105.5 (C-2, α-fruf), 102.6 (C-2, β-fruf), 99.2 (C-2, β-frup), 97.0 (C-1, β-glcp), and 93.2 (C-1, α-glcp), were selected to estimate their concentration and their relative proportion. However, in order to eliminate the problem of different levels of NOE enhancement and dissimilar relaxation properties of these carbon atoms, the relative proportion of fructose to glucose was not calculated from the ratio of integrals; instead the concentration of the two hexoses was estimated using the standard addition method. On the basis of their concentrations, the relative proportion of fructose to glucose in the original nectar of *A. flavidus* was then calculated to be 1.0:1.2 ± 0.05. ^{13}C NMR measurement of an artificial validation sample, which contained the two hexoses exactly in the native ratio, confirmed this result (Figure 2).

Out of the three nectars used in this study, only that of *A. manglesii* has been previously analyzed for its relative carbohydrate composition. A ratio of fructose to glucose to sucrose of 40:55:5 has been determined by quantitative paper chromatography.[8] Our study confirmed that the nectar of *A. manglesii* and the two other *Anigozanthos* nectars are largely hexose-dominated. However, as mentioned above, the sucrose concentration was too low to be detectable by ^{13}C NMR. In order to rule out the possibility of sucrose hydrolysis after nectar collection due to acid pH or invertase activity,[27,28] some samples were measured immediately after the nectar was collected from the flower, but sucrose was detected (by ^1H NMR) at the same low level as in nectar stored at −20 °C. Hence, the absence of ^{13}C NMR-detectable levels of sucrose in *Anigozanthos* nectar suggested invertase-catalyzed hydrolysis occurred before sucrose was secreted to the nectar.

According to our results, the relative carbohydrate composition determined for *A. flavidus* was roughly the same in nectar samples of the two other *Anigozanthos* species, *A. manglesii* and *A. humilis*. It is interesting that a slight excess of glucose over fructose has also been reported for other passiflorine-pollinated plants, e.g., *Strelitzia reginae*,[29] and several species.[8] The observed fructose to glucose ratio of 1.0:1.2 cannot be explained by the simple action of an invertase. It would be interesting to know how the relative proportions of the glucose to fructose and of sucrose to hexoses are regulated and in which particular cells of the nectary the sugar conversions take place. Current knowledge about invertases, which are involved in nectar production,[27] aspects of nectar origin,[30] and the genetic basis of nectar production[31] have been reviewed recently. The results of the present NMR study can be used to support physiological, biochemical, and molecular studies in this field.

Unlike relative sugar proportions, nectar amounts in the flowers and carbohydrate concentrations seemed to not be constant but varied depending on the different times of the day and external conditions (e.g., temperature, light intensity, relative air humidity) under which samples were collected. As shown in Table 1, the estimated concentration in freshly collected samples of *A. flavidus* nectar was around 455 mmol L^{-1} fructose and 540 mmol L^{-1} glucose. However, no attempts were made to analyze in detail daily fluctuation or the effect of external parameters on the sugar content

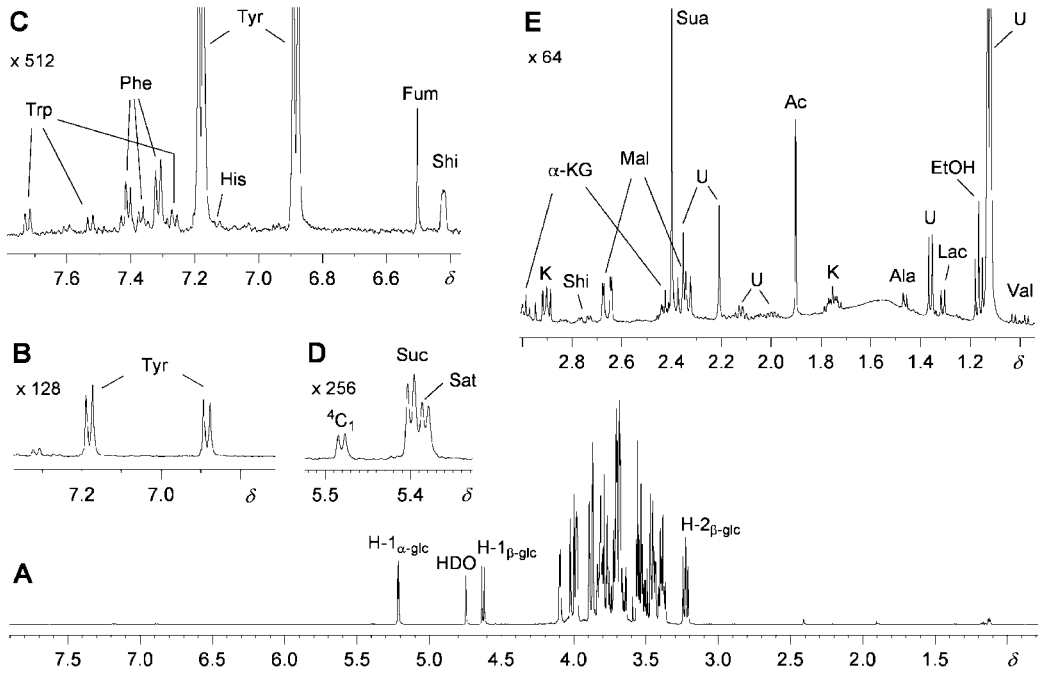

Figure 3. ^{1}H NMR spectrum (500 MHz, phosphate buffer pH 6.5) (A) and magnified partial spectra (B−E, numbers indicate vertical magnification) of floral nectar (50 μL) of *Anigozanthos flavidus*. Compounds were identified and quantified by means of adding standards. Spectrum A shows signals of the major components, glucose and fructose; HDO indicates the residual water signal remaining after applying PURGE.[25] Ac, acetic acid; Ala, alanine; $^4C_1$, $^4C_1$-conformer of α-D-glucopyranose; EtOH, ethanol; Fum, fumaric acid; His, histidine; K, contamination from buffer; α-KG, α-ketoglutaric acid; Lac, lactic acid; Mal, malic acid; Phe, phenylalanine; Shi, shikimic acid; Sat, ^{13}C satellite signal of H-1 of α-D-glucopyranose; Sua, succinic acid; Suc, sucrose; Trp, tryptophan; Tyr, tyrosine; U, unknown; Val, valine.

of nectar samples. Just as nectar composition in other species[12,13] and the effects of various environmental factors vary,[32,33] so do the levels of glucose and fructose given in Table 1 for *A. flavidus.*

Minor Components of *Anigozanthos* Nectar. Since in the ^{13}C NMR spectra only glucose and fructose were observed, the levels of other sugars and other nectar components were expected to be lower by at least 2 orders of magnitude, necessitating a more sensitive analytical method. ^{1}H NMR using a cryogenically cooled probe was the method of choice because it is considerably more sensitive than ^{13}C NMR. The first carbohydrate candidate we looked for was sucrose because, surprisingly, it was not found by ^{13}C NMR in the nectar of *A. flavidus*, although it is usually one of the major constituents in floral nectars. In addition to the large doublets of H-1$_{\alpha\text{-glc}}$ (δ 5.215, J = 3.8 Hz) and H-1$_{\beta\text{-glc}}$ (δ 4.628, J = 8.0 Hz), the ^{1}H NMR spectrum exhibited two doublets attributable to the protons at the anomeric center of hexopyranoses. Adding a defined amount of sucrose, we identified the doublet at δ 5.400 (J = 3.8 Hz) as the anomeric H-1α of sucrose (Figure 3D) and estimated its concentration to be 1.4 mmol L^{-1}. Due to the coupling constant of J = 3.8 Hz and the occurrence in the ^{1}H NMR spectrum of the glucose standard, the doublet at δ 5.481 (Figure 3D) was assigned to H-1 of the energetically disfavored $^4C_1$-conformer of α-D-glucose (integral 0.003% of the central doublet of H-1$_{\alpha\text{-glc}}$). Alternatively, this signal might be assignable to an unidentified α-sugar or α-configured unit of a di- or oligosaccharide, although further signals of additional sugar components were not detected. However, the crowded spectral region δ 4.1–3.2, the doublets of H-1$_{\alpha\text{-glc}}$ and H-1$_{\beta\text{-glc}}$, and saturation of the HDO signal (Figure 3A) may obscure signals of minor carbohydrates or carbohydrate derivatives such as sugar acids, amino sugars, and sugar phosphates; however, no references were available for such hypothetical nectar components. A ^{31}P NMR spectrum was recorded to check for the occurrence of phosphorus compounds, e.g., sugar phosphates, in *Anigozanthos* nectar. The spectrum showed a small signal, which on the basis of an added standard, was attributed to phosphate.

Amino acids have been detected in most floral nectars investigated.[6] After carbohydrates, they are the second most abundant class of compounds found in nectar.[9] ^{1}H NMR seemed promising for its ability to detect minor sugars and also to search for amino acids and other minor components. Therefore, nectar samples were measured by ^{1}H- and ^{1}H-detected 2D NMR methods in deuterated K-Pi buffer pH 6.5. As expected, the ^{1}H NMR spectrum (Figure 3A) displays major carbohydrate signals between δ 5.3 and 3.2. Magnification of the ^{1}H NMR spectrum revealed additional signals. The two doublets (J = 8.0 Hz) shown in Figure 3B are assignable to an AXA′X′ spin system of a *p*-substituted phenyl ring. The only amino acid having that structural moiety is tyrosine, from which an ^{1}H NMR spectrum was measured under identical conditions in order to compare spectra directly. A complete match of the fingerprint signals of nectar samples and the authentic standard proved the occurrence of tyrosine in the floral nectar of *A. flavidus*. Cross-peaks of the tyrosine side chain [H-2 (δ 3.920)−H-3a (δ 3.189) and H-2−H-3b (δ 3.038)] in the ^{1}H−^{1}H COSY and correlations in the long-range COSY (lrCOSY) spectrum [H-2′/6′ (δ 7.181)−H-3a and H-2′/6′−H-3b] measured from an *A. flavidus* nectar sample further confirmed this finding. The concentration of tyrosine, estimated by the standard addition method, was 15.0 mmol L^{-1} in *A. flavidus* nectar. ^{1}H NMR analysis of nectar samples of *A. manglesii* and *A. humilis* resulted in similar tyrosine levels (data not shown). Inspecting the low-field part of the vertically extended ^{1}H NMR spectrum (Figure 3C) for signals of further amino acids and other minor components and applying the standard addition method for quantification, we established the presence of low levels of phenylalanine (250 μmol L^{-1}), tryptophan (80 μmol L^{-1}), histidine (30 μmol L^{-1}), fumaric acid (110 μmol L^{-1}), and shikimic acid (100 μmol L^{-1}). Chemical shifts of characteristic signals used for identification and quantification are given in Table 1. Two aliphatic amino acids, alanine (120 μmol L^{-1}) and valine (60 μmol L^{-1}), were detected from their methyl signals in the high-field region of the ^{1}H NMR spectrum by means of their characteristic coupling

Table 1. Concentration and NMR Data Used for Identification and Quantification of *Anigozanthos flavidus* Nectar Components

compound	NMR method	NMR signals used for identification and quantification δ (mult., J in Hz, assignment)	reference NMR signal[a] metabolite, δ	concentration mmol L^{-1}
Carbohydrates				
fructose[b]	^{13}C	105.5 (C-$2_{\alpha\text{-fruf}}$); 102.6 (C-$2_{\beta\text{-fruf}}$); 99.2 (C-$2_{\beta\text{-frup}}$)	β-glcp, 97.0	455
glucose[c]	^{13}C	97.0 (C-$1_{\beta\text{-glcp}}$); 93.2(C-$1_{\alpha\text{-glcp}}$)	β-frup, 99.2	540
sucrose	^{1}H	5.400 (d, 3.8, H-1_{glc})	Tyr, 6.885	1.40
Amino acids				
alanine	^{1}H	1.464 (d, 7.2, CH_3)	U[d], 2.210	0.12
histidine	^{1}H	7.989 (s, H-2′); 7.138 (s, H-5′)	Tyr, 6.885	0.03
phenylalanine	^{1}H	7.416 (m, H-3′/5′); 7.363 (m, H-4′); 7.314 (d, 8.0, H-2′/6′)	Tyr, 6.885	0.25
tryptophan	^{1}H	7.723 (d, 8.1, H-4); 7.527 (d, 8.3, H-7); 7.272 (dd, 8.3, 8.0, H-6);	Tyr, 6.885	0.08
tyrosine	^{1}H	7.181 (d, 8.5, H-2′/6′); 6.885 (d, 8.5, H-3′/5′)	Tyr, 6.885	15.0
valine	^{1}H	1.027 (d, 7.0, CH_3-4′); 0.976 (d, 7.0, CH_3-4)	U, 2.210	0.06
Carboxylic acids				
acetic acid	^{1}H	1.902 (s, CH_3)	U, 2.210	1.20
fumaric acid	^{1}H	6.504 (s, CH)	Tyr, 6.885	0.11
α-ketoglutaric acid	^{1}H	2.986 (t, 7.0, H_2-4); 2.428 (t, 7.0, H_2-3)	U, 2.210	0.27
lactic acid	^{1}H, 2D	1.312 (d, 7.0, CH_3)	U, 2.210	0.26
malic acid	^{1}H	2.660 (dd, 15.4, 2.9, H-3a); 2.350 (dd, 15.4, 10.3, H-3b)	U, 2.210	4.70
shikimic acid	^{1}H	6.42 (brs, H-2); 2.750 (dd, 18.0, 5.5, H-6a)	Tyr, 6.885	0.10
succinic acid	^{1}H	2.400 (s, CH_2)	U, 2.210	2.00
Alcohols				
ethanol	^{1}H	1.168 (t, 7.1, CH_3)	U, 2.210	1.00
Secondary metabolites				
diarylheptanoid	^{1}H, 2D	see Experimental Section		

[a] This signal was used for normalizing the spectra in the standard addition method. [b] Equilibrium mixture of β-D-fructopyranose, β-D-fructofuranose, and α-D-fructofuranose. [c] Equilibrium mixture of α-D-glucopyranose and β-D-glucopyranose. [d] U, unknown nectar component.

constants ($J \approx 7$ Hz) (Figure 3E). Malic acid (doublets of doublets at δ 2.660 and 2.350) and succinic acid (singlet at δ 2.400) occurred at relatively high levels (4.7 and 2.0 mmol L^{-1}), respectively. Further signals in the high-field region were assigned to α-ketoglutaric acid (270 μmol L^{-1}), acetic acid (1.2 mmol L^{-1}), and ethanol (1.0 mmol L^{-1}). A tiny doublet of doublets at δ 2.750 was attributable to the H-6a of shikimic acid, which was better detected from its characteristic low-field signal of H-2 (δ 6.422). It is interesting to note that four organic acids, each part of the citric acid cycle, namely, fumaric acid, α-ketoglutaric acid, malic acid, and succinic acid, were detected.

Supplementing a nectar sample with threonine or lactic acid in either case enhanced the methyl doublet at δ 1.312 in the ^1H NMR spectrum (Figure 4A) and therefore did not allow this signal to be assigned to one of the two compounds. Since other proton resonances of lactic acid and threonine would be obscured by large glucose and sucrose signals, 1D ^1H NMR could not be used to determine this component. Hence, a sample containing 400 μL of nectar was subjected to 2D NMR experiments ($^1H-^1H$ COSY, HSQC, HMBC). According to the homo- and heteronuclear correlations, which were detected by these experiments, the doublet at δ 1.312 is assignable to the methyl group of lactic acid but not threonine. For example, the partial HMBC spectrum (Figure 4B) exhibits $^1H-^{13}C$ cross signals due to the long-range correlation of the methyl protons with C-2 (δ 69.3) and the carboxyl group (δ 183.4). The signals observed in the nectar sample by ^1H NMR and 2D NMR methods matched those of authentic lactic acid. Cross-peaks, which would have been expected for correlations between the methyl group and C-2 (δ 61.0) and C-3 (δ 66.6) of threonine, were not observed. Quantification, again using the standard addition method, resulted in a value of 260 μmol L^{-1} lactic acid in nectar of *A. flavidus*.

Although periodic changes in the levels of minor components were not investigated systematically—as they were not in carbohydrates—some variations were detected in samples collected on different days. Possible intraplant variation was not observed because nectar was pooled from a multitude of individual flowers (average content 15 μL nectar per flower). The minor differences observed are assumed to be due to variations of temperature and other external conditions. In general, however, the overall composition of samples was relatively stable.

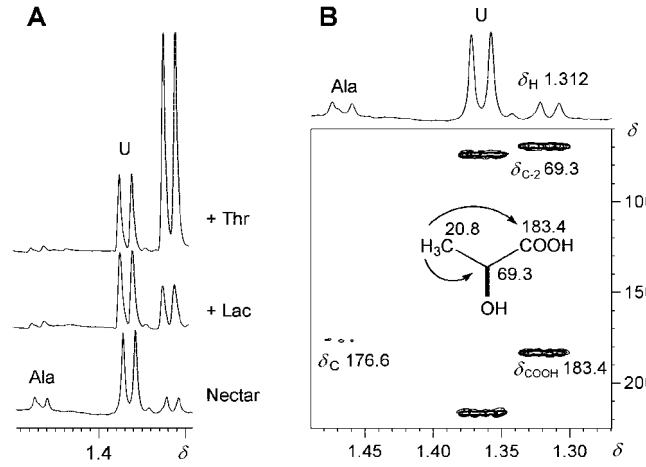

Figure 4. NMR spectroscopic identification of lactic acid in nectar of *Anigozanthos flavidus*. (A) Partial ^1H NMR spectra (500 MHz) of a nectar sample (50 μL in K-Pi buffer pH 6.5) (bottom), nectar supplemented with lactic acid (middle), and threonine (top). (B) Partial HMBC spectrum (500 MHz) of a nectar sample (400 μL/100 μL K-Pi buffer pH 6.5). Cross-signals of δ_{CH3} 1.312 with $\delta_{C\text{-}2}$ 69.3 and δ_{COOH} 183.4 are consistent with lactic acid but not with threonine (expected cross-signals with $\delta_{C\text{-}2}$ 61.0 and $\delta_{C\text{-}3}$ 66.6). The doublet at $\delta_{\underline{CH}3}$ 1.464 and the weak cross-signal δ_{COOH} 176.6 are due to alanine. The signal labeled with "U" is from an unidentified compound.

The ecological role of amino acids and other minor nectar components remains uncertain. Tyrosine does not belong to the group of amino acids that are essential to the nutrition of insects.[15] Hence, the occurrence of relatively high levels of this amino acid in *Anigozanthos* floral nectars suggests that the plant is not pollinated by insects. Instead, tyrosine might be essential to honeyeaters (Meliphagidae), which are reported to be the major pollinators of *Anigozanthos* flowers.[1,2] The much lower levels of other amino acids suggest that only tyrosine is a significant nitrogen source.[34] Perception studies of nectar consumers are needed to learn more about the importance of tyrosine and the other minor components in the floral nectar.

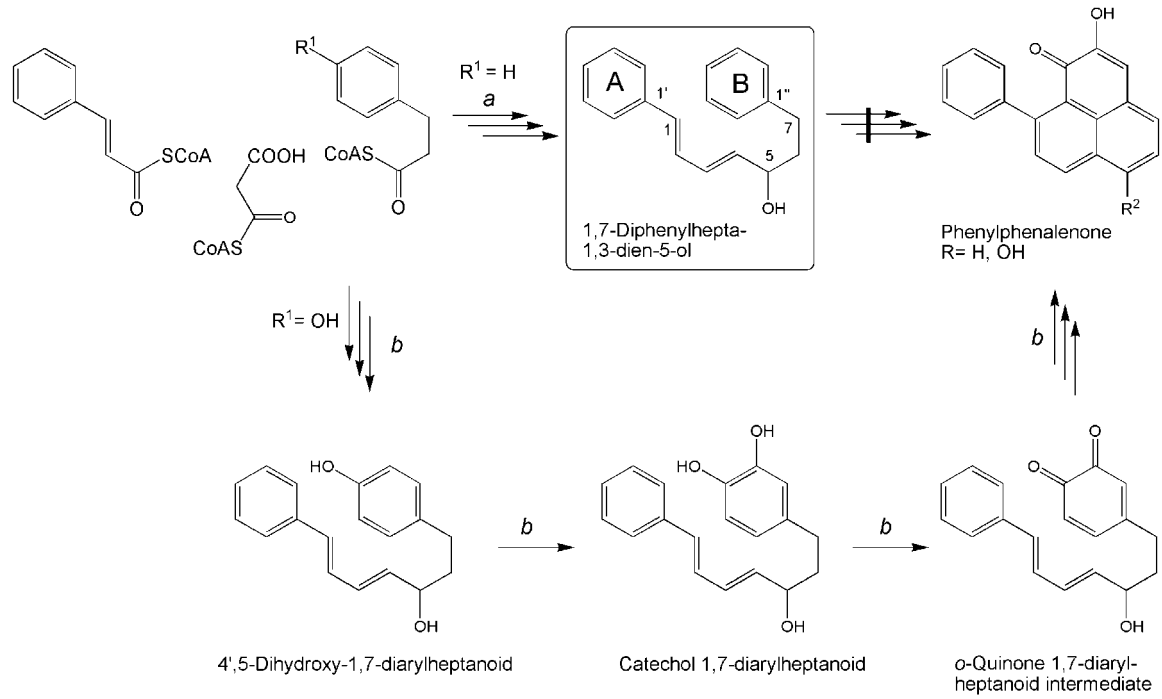

Figure 5. Biosynthesis scheme illustrating the formation through path *a* of 1,7-diphenylheptan-1,3-dien-5-ol, which has been identified as a final product of the diarylheptanoid pathway in floral nectar of *Anigozanthos* species. In contrast, detectable levels of linear diarylheptanoids having oxygen functions at phenyl ring B do not accumulate in *Anigozanthos* but, through path *b* including a [4 + 2]-cyclization as a key step, finally form phenylphenalenones.[42,43]

Trace Components: A Secondary Product from *Anigozanthos* Nectar. In addition to carbohydrates and amino acids, lipids,[35] and volatiles,[10,36] a number of secondary products have been reported as nectar components.[7]Secondary metabolites may have attractive or repellent characteristics.[10,37] Our interest focused on phenylphenalenones, which are known constituents of *Anigozanthos* and other Hemodoraceae.[38] Further compounds of interest include diarylheptanoids; although these have been shown to be biogenetic precursors of phenylphenalenones[39] in Hemodoraceae, they have never been found in any plant of this family. The signals of these compounds were not observed in the ^{13}C and ^{1}H NMR spectra obtained from nectar samples. However, secondary metabolites might occur in traces only and require enrichment before NMR detection is possible. Hence, a nectar sample (7.5 mL) was collected and pooled from flowers of *A. flavidus*. The sample was passed through an RP-18 cartridge to remove the major amounts of carbohydrates, amino acids, and polar organic acids and to enrich lipophilic components. Despite this purification step, carbohydrates were not completely removed from the sample and still represented major signals in the ^{1}H NMR spectrum of that nectar fraction. Neither comparisons of this spectrum to ^{1}H NMR spectra of a multitude of phenylphenalenones available in our database nor comparisons to the literature hinted at the presence of such compounds in the sample.

However, three doublets of doublets at δ 6.89 (J = 15.5, 10.8 Hz), 5.86 (J = 15.2, 6.2 Hz), and 6.38 (J = 15.2 and 10.8 Hz) and a doublet resonating at δ 6.64 (J = 15.5 Hz) suggested a compound containing two conjugated *trans* double bonds. This structural feature is typical for many linear diarylheptanoids and was confirmed by a series of cross-peaks in the $^{1}H-^{1}H$ COSY spectrum [H-1 (δ 6.64)−H-2 (δ 6.89)−H-3 (δ 6.38)−H-4 (δ 5.86)] of this sample. The multiplicity of H-4 (doublet of doublets) strongly suggested a proton of an adjacent methine group (H-5). Another correlation in the COSY spectrum was assigned to two adjacent methylene groups, H_2-7 (δ 2.60)−H_2-6 (δ 1.90). Furthermore, an lrCOSY spectrum established the attachment of two unsubstituted phenyl rings to the terminal positions of the C-7 chain. On the basis of these data, the structure was tentatively assigned to be (*E,E*)-1,7-diphenylhepta-1,3-dien-5-ol, a diarylheptanoid first isolated from *Curcuma xanthorrhiza*.[40] LC-ESIMS (*m/z* 247 [(M + H) − H_2O]$^+$) confirmed the molecular mass. Furthermore, (*E,E*)-1,7-diphenylhepta-1,3-dien-5-ol was synthesized according to the Wittig–Horner approach used for preparing other diarylheptanoids.[41] The NMR data of the synthetic sample matched those of the compound isolated from *Anigozanthos* nectar. Using the same approach, this diarylheptanoid was also found in the nectar of *A. manglesii*. Unfortunately the amount of floral nectar obtained from *A. humilis* was insufficient to analyze trace components.

This compound is the first diarylheptanoid to be reported from a Hemodoraceae species. Although plants and *in vitro* root cultures of *A. flavidus* and other members of this genus have been studied in some detail, diarylheptanoids have not been detected. This prompted us to reinvestigate *A. flavidus* for diarylheptanoids. Crude extracts and fractions prepared by solid-phase extraction of the roots, above-ground plant material, and inflorescences were checked by reversed-phase HPLC using synthetic (*E,E*)-1,7-diphenylhepta-1,3-dien-5-ol and other synthetic diarylheptanoids[41] as authentic references (data not shown). Although the occurrence of small levels of diarylheptanoids cannot be fully excluded by such experiments, compounds of that type were not detected and definitely are not among the major constituents of *Anigozanthos*.

The identification of (*E,E*)-1,7-diphenylhepta-1,3-dien-5-ol in the nectar and the failure to detect this or other diarylheptanoids in the plant material deserves some biosynthetic consideration. The biosynthetic hypothesis[42,43] (Figure 5) suggested that, in the plant cell, an *o*-quinone structure at one of the aryl rings of the diarylheptanoid is required for cyclization. The *o*-quinone, which is thought to be formed by oxidation of a catechol, is considered an ideal dienophile with which to form phenylphenalenones via an intramolecular Diels–Alder cyclization with the diene moiety of the C-7 chain.[42] Hence, even if the enzymes for such cyclization were active in the nectary or the nectar, the formation of phenylphenalenones seems to be impossible if the phenyl ring attached to C-7 lacks the oxygen functions at C-3″ and C-4″. However, further investigation is needed

to confirm this hypothesis. Whether or not (*E,E*)-1,7-diphenylhepta-1,3-dien-5-ol is involved in ecological interactions between *Anigozanthos* and visitors of its flowers remains to be studied.

Conclusions

Various amino acids, organic acids, and other components, in addition to major carbohydrates, have been detected in the floral nectar of *A. flavidus* and also in two other species of this genus. This study demonstrates that different NMR spectroscopic techniques are suitable for the characterization of nectar samples and the determination of individual components over a wide range of concentrations (30 μmol L^{-1} histidine to approximately 0.5 mol L^{-1} glucose) with a minimum of sample preparation and without derivatization. While ^{13}C NMR is useful for identifying major carbohydrates, cryogenic ^1H and 2D NMR techniques are needed to analyze minor components, such as amino acids and trace compounds. The NMR methodology we used is especially appropriate for the metabolic profiling of nectar samples in ecological studies.

The example of a diarylheptanoid, which has been detected and identified in a fraction obtained by solid-phase extraction of the crude nectar but not in other parts of the *Anigozanthos* plant, shows the specific occurrence of secondary metabolites, though present in trace amounts, in nectar. Moreover, detection of the first linear diarylheptanoid in the Hemodoraceae, a family characterized by the occurrence of phenylphenalenones, inspired biosynthetic consideration of the role of the oxygenation pattern in cyclization and aromatization, both of which are essential steps of phenylphenalenone formation.

Experimental Section

General Experimental Procedures. An Avance 500 spectrometer (Bruker, Rheinstetten, Germany) equipped with a 5 mm TCI cryoprobe was used for measuring ^1H NMR spectra (500 MHz), 2D homo- and heteronuclear correlation NMR spectra ($^1H-^1H$ COSY, lrCOSY, HSQC, HMBC), and ^{13}C NMR spectra (125 MHz; power gated decoupling; 30° flip angle; relaxation delay = 2 s, line broadening factor = 1) of nectar samples. The PURGE sequence[25] was used to suppress the water signal in ^1H NMR spectra. 3-(Trimethylsilyl)propionic acid-d_4 (TSP, δ_H −0.01) was used as an internal standard for referencing ^1H NMR spectra. ^{31}P NMR spectra (162 MHz) of nectar and ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra of synthetic compounds were measured using an Avance 400 spectrometer (Bruker) equipped with a 5 mm broadband room-temperature probe. Tetramethylsilane (TMS) was used as an internal standard for referencing ^1H NMR and ^{13}C spectra. ^{31}P spectra remained unreferenced. All NMR spectra were run at 30 °C. Mass spectra (EIMS and HREIMS) were recorded on a MasSpec sector field mass spectrometer (Micromass Ltd., Manchester, UK) with a direct insertion probe. Electrospray mass spectra (LC-ESIMS) were recorded using a Micromass Quattro II (Micromass) tandem quadrupole mass spectrometer equipped with an electrospray source.

Plant Material and Sample Collection. Plants of *Anigozanthos flavidus* DC., *Anigozanthos manglesii* D. Don subsp. *manglesii,* and *Anigozanthos humilis* subsp. *chrysanthus* Hopper were raised from seeds (Chiltern Seeds, Bortree Stile, UK) and grown in soil in the greenhouse (day 20–24 °C, night 18–21 °C; relative air humidity 60–70%; the natural daily photoperiod was supported by 16 h illumination from Phillips Sun-T Agro 400 Na lights). Nectar samples were collected in July and August from freshly opened flowers between 9 and 10 a.m. Samples were pooled in amounts of approximately 1 mL and, if not analyzed immediately, stored in a freezer at −20 °C. The average content was approximately 15 μL of nectar per flower.

Sample Preparation and NMR Conditions. Nectar samples, freshly collected or stored at −20 °C, were used for NMR analyses. Carbohydrate analysis: Floral nectar samples (50 μL) of *A. flavidus, A. manglesii,* and *A. humilis* were dissolved in 450 μL of deuterated K-Pi buffer (1 mmol L^{-1}, pH 7.5) for recording ^1H and ^{13}C NMR spectra. Minor constituents (e.g., amino acids and carboxylic acids): If not otherwise mentioned in the text, floral nectar samples (50 μL) were dissolved in 450 μL of deuterated K-Pi buffer (1 mmol L^{-1}, pH 6.5) for measuring ^1H and 2D NMR spectra. For quantitative analysis, a solution (10 μL) of a defined amount of the standard in K-Pi buffer (1 mmol L^{-1}, pH 6.5) was added to the nectar sample before the ^1H NMR measurement was repeated under otherwise identical conditions. A sample prepared from 400 μL of nectar and 100 μL of D_2O was used for ^{31}P NMR spectra (ns 64K). Trace components: Floral nectar of *A. flavidus* (7.5 mL) and *A. manglesii* (2.5 mL), respectively, was dissolved in H_2O (20 mL) and passed through an RP-18 cartridge (100 mg). The cartridge was flushed with water (20 mL) and eluted with MeCN (1 mL). The MeCN solution was evaporated and the residue dissolved in DMSO-d_6 (99.8%) followed by ^1H NMR, $^1H-^1H$ COSY, and lrCOSY analysis.

(*E,E*)-1,7-Diphenylhepta-1,3-dien-5-ol from floral nectar of *A. flavidus*: ^1H NMR (DMSO-d_6, 500 MHz) δ 7.45 (2H, d, J = 8.0 Hz, H-2′/6′), 7.31 (2H, m, H-3′/5′), 7.27 (2H, m, H-3″/5″), 7.21 (1H, m, H-4′), 7.19 (2H, m, H-2″/6″), 7.17 (1H, m, H-4″), 6.89 (1H, dd, J = 15.5, 10.8 Hz, H-2), 6.64 (1H, d, J = 15.5 Hz, H-1), 6.38 (1H, dd, J = 15.2, 10.8 Hz, H-3), 5.86 (1H, dd, J = 15.2, 6.2 Hz, H-4), 2.60 (2H, m, H-7), 1.90 (2H, m, H-6); H-5 and OH-5 not detected; LC-ESIMS *m/z* 247 $[(M + H) - H_2O]^+$; daughter ion scan (12 eV) *m/z* 124; daughter ion scan (25 eV) *m/z* 143, 128, 91.

Synthesis of (*E,E*)-1,7-Diphenylhepta-1,3-dien-5-ol. (*E,E*)-1,7-Diphenylhepta-1,3-dien-5-one (alnustone) was synthesized according to the Wittig–Horner method as described for other diarylheptadienones.[41] ^1H NMR ($CDCl_3$, 400 MHz) δ 7.46 (2H, d, J = 8.0 Hz, H-2′/6′), 7.35 (2H, m, H-3′/5′), 7.32 (1H, m, H-4′), 7.32 (1H, dd, J = 15.5, 10.1 Hz, H-3), 7.29 (2H, m, H-3″/5″), 7.22 (2H, d, J = 8.0 Hz, H-2″/6″), 7.20 (1H, m, H-4″), 6.93 (1H, d, J = 15.5 Hz, H-1), 6.87 (1H, dd, J = 15.5, 10.1 Hz, H-2), 6.28 (1H, d, J = 15.5 Hz, H-4), 2.97 (2H, m, H-7), 2.93 (2H, m, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 199.4 (C-5), 142.7 (C-3), 141.4 (C-1), 141.3 (C-1″), 136.0 (C-1′), 129.5 (C-4), 129.2 (C-4′), 128.8 (C-2″/6″), 128.5 (C-3′/5′), 128.4 (C-3′/5′), 127.2 (C-2′/6′), 126.7 (C-2), 126.1 (C-4″), 42.3 (C-6), 30.2 (C-7); EIMS *m/z* 263 $[M + H]^+$, 262 $[M]^+$, 171, 157, 128, 115, 105, 91 (100%), 77, 65; HREIMS *m/z* 262.13538 (calc for $C_{19}H_{18}O$, 262.13577).

(*E,E*)-1,7-Diphenylhepta-1,3-dien-5-one (77 μmol, 20.2 mg) was dissolved in MeOH (1 mL) and cooled to 0 °C. $NaBH_4$ (80 μmol, 3 mg) was added and the mixture stirred at 0 °C for 30 min. The reaction was stopped by adding acetone (1 mL). After evaporation the residue was taken up in $CHCl_3$ (5 mL), filtered, and evaporated to yield 15 mg (57 μmol, 73%) of (*E,E*)-1,7-diphenylhepta-1,3-dien-5-ol: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.46 (2H, d, J = 8.0 Hz, H-2′/6′), 7.32 (2H, m, H-3′/5′), 7.27 (2H, m, H-3″/5″), 7.24 (2H, m, H-2″/6″), 7.22 (1H, m, H-4′), 7.16 (1H, m, H-4″), 6.92 (1H, dd, J = 15.4, 10.8 Hz, H-2), 6.59 (1H, d, J = 15.4 Hz, H-1), 6.42 (1H, dd, J = 15.2, 10.8 Hz, H-3), 5.94 (1H, dd, J = 15.2, 6.2 Hz, H-4), 4.20 (1H, m, H-5), 3.97 (1H, d, J = 4.7 Hz, OH-5), 2.75 (2H, m, H-7), 1.83 (2H, m, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 143.1 (C-1′), 139.4 (C-4), 138.4 (C-1″), 132.6 (C-1), 130.6 (C-5), 129.9 (C-6), 129.6 (C-3′/5′), 129.4 (C-2″/6″), 129.3 (C-3″/5″), 128.4 (C-4′), 127.3 (C-2′/6′), 126.7 (C-4″), 71.4 (C-3), 40.5 (C-2), 32.6 (C-1); ^1H NMR (DMSO-d_6, 500 MHz) δ 7.44 (2H, d, J = 8.0 Hz, H-2′/6′), 7.31 (2H, m, H-3′/5′), 7.27 (2H, m, H-3″/5″), 7.19 (2H, m, H-2″/6″), 7.21 (1H, m, H-4′), 7.16 (1H, m, H-4″), 6.90 (1H, dd, J = 15.4, 10.8 Hz, H-2), 6.56 (1H, d, J = 15.4 Hz, H-1), 6.34 (1H, dd, J = 15.2, 10.8 Hz, H-3), 5.90 (1H, dd, J = 15.2, 6.2 Hz, H-4), 4.04 (1H, m, H-5), 4.93 (1H, d, J = 4.8 Hz, OH-5), 2.63 (2H, m, H-7), 1.72 (2H, m, H-6); EI-MS *m/z* 265 $[M + H]^+$, 264 $[M]^+$, 159, 133, 128, 115, 105, 91 (100%), 77, 65; HRMS *m/z* 264.15232 (calc for $C_{19}H_{20}O$, 264.15142). The NMR data of (*E,E*)-1,7-diphenylhepta-1,3-dien-5-ol matched those reported for the previously synthesized compound[44] and the natural product isolated from *C. xanthorrhiza*.[40]

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