The Biosynthesis of Branched Dialkylpyrazines in Myxobacteria

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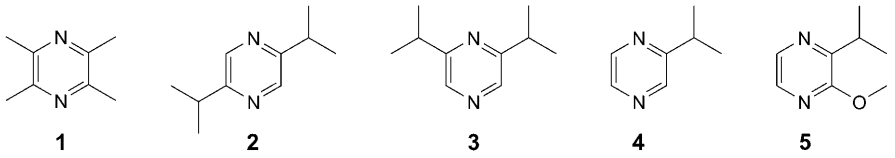
The biosynthesis of the volatiles 2,5- and 2,6-diisopropylpyrazine (**2** and **3**, resp.) released by the myxobacteria *Nannocystis exedens* subsp. *cinnabarina* (Na c29) and *Chondromyces crocatus* (strains Cm c2 and Cm c5) was studied. Isotopically labeled precursors and proposed pathway intermediates were fed to agar plate cultures of the myxobacteria. Subsequently, the volatiles were collected by use of a closed loop stripping apparatus (CLSA), and incorporation into the pyrazines was followed by GC/MS analysis. [$^2H_8$]Valine was smoothly incorporated into both pyrazines clearly establishing their origin from the amino acid pool. The cyclic dipeptide valine anhydride (**16**) – a potential intermediate on the biosynthetic pathway to branched dialkylpyrazines – was synthesized containing $^2H_1$ labels in specific positions. Feeding of [$^2H_{16}$]-**16** and [$^2H_{12}$]-**16** in both valine subunits mainly resulted in the formation of pyrazines derived from only one labeled amino acid, whereas only traces of the expected pyrazines with two labeled subunits were found. To investigate the origin of nitrogen in the pyrazines, a feeding experiment with [^{15}N]valine was performed, resulting in the incorporation of the ^{15}N label. The results contradict a biosynthetic pathway *via* cyclic dipeptides, but rather point to a pathway on which valine is reduced to valine aldehyde. Its dimerization to 2,5-diisopropyldihydropyrazine **36** and subsequent oxidation results in **2**. The proposed biosynthetic pathway neatly fits the results of earlier labeling studies and also explains the formation of the regioisomer 2,6-diisopropylpyrazine **3** by isomerization during the first condensation step of two molecules valine aldehyde. A general biosynthetic pathway to different classes of pyrazines is presented.

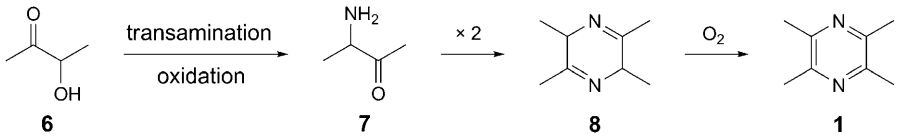
Introduction. – Pyrazines are important odoriferous compounds that play a major role in aroma and taste formation [1][2]. They are formed by the *Maillard* reaction which takes place during heating of amino acids and carbohydrates [3][4]. Apart from this chemical formation, pyrazines are also produced by many bacteria as volatiles or during fermentation of food [1][2][5]. The naturally occurring pyrazines can be divided into three different classes. The first class constituted by pyrazines with up to four alkyl substituents, predominately Me or Et groups as in 2,3,5,6-tetramethylpyrazine (**1**), whereas longer side chains are rare. They are frequently found in bacteria such as *Corynebacterium glutamicum* that produces alkylated pyrazines in high amounts [5–7]. Proof for the involvement of bacteria in the production of pyrazines is sometimes difficult, because especially the simple members of this class such as 2,5-dimethylpyrazine can be formed during autoclavation of culture media [8]. The second class of pyrazines consists of compounds containing two branched side chains as represented by 2,5- and 2,6-diisopropylpyrazine (**2** and **3**, resp.) that are obviously derived from the amino acids valine, leucine, or isoleucine [9]. Pyrazines with only one branched alkyl group in the 2-position and, optionally, a Me or Et substituent in the 5- or 6-position,

e.g., 2-isopropylpyrazine (**4**), occur also [5]. Finally, methoxypyrazines constitute the third pyrazine class that usually carry one or two alkyl substituents as found in 2-methoxy-3-isopropylpyrazine (**5**) [5].



Only few efforts have been made so far to investigate the biosynthesis of pyrazines in bacteria [2][9–15]. Recently, we have shown by a combination of gene knockout and feeding experiments that the biosynthetic pathway to the first class of pyrazines in *C. glutamicum* includes a sequence of transamination and oxidation of acetoin (= 3-hydroxybutan-2-one; **6**) and its higher homologues to α-amino ketones, followed by condensation and oxidation [7] (*Scheme 1*).

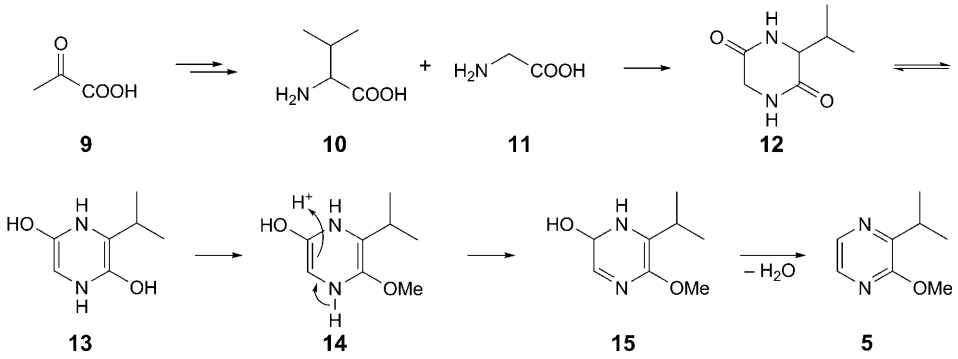
Scheme 1. *Biosynthesis of Tetramethylpyrazine* (**1**) [7]



2-Alkyl-3-methoxypyrazines often have a low odor threshold and are characteristic aroma components of different foods like peas or boiled potatoes [10][16]. These compounds were initially proposed to be formed by the condensation of an amino acid amide and glyoxal, followed by *O*-methylation [10]. Later, *Cheng et al.* suggested the formation of **5** by a process involving condensation of valine (**10**) and glycine (**11**) to form the piperazinedione **12** that, upon elimination of H_2O, would yield a hydroxypyrazine. Methylation during this sequence would lead to the methoxypyrazine **5** (*Scheme 2*). Feeding studies with labeled valine (**10**) and glycine (**11**) established the incorporation of the former into **5**, but glycine (**11**) was not incorporated [12], possibly because it was metabolized before the pyrazine formation started [13]. To overcome this problem, feeding experiments with differently ^{13}C-labeled pyruvate isotopomers as metabolic precursors of glycine and valine were carried out, supporting the proposed bacterial pathway to the third class of pyrazines [13]. An identical mechanism was proposed for the formation of 2-methoxy-3-(1-methylpropyl)pyrazine in *Halomonas venusta* that showed increased formation of the respective piperazinedione intermediate when glycine and isoleucine were supplied [15].

Pyrazines of the second type were only recently found to be produced by bacteria [1], and the biosynthetic pathway to these compounds is unknown. 2,5-Diisopropylpyrazine (**2**) is the major pyrazine produced by *Paenibacillus polymyxa*. The addition of valine (**10**) to the culture medium led to an increased production of **2** and an inhibition

Scheme 2. *Proposed Biosynthetic Pathway to 3-Isopropyl-2-methoxypyrazine According to* Cheng et al. [13]



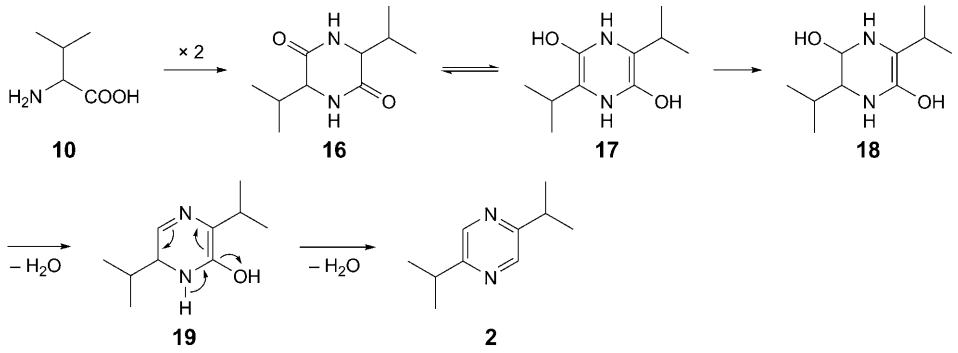
of the formation of all other pyrazines otherwise produced by this bacterium [9]. During the course of our program on the investigation of volatiles released by myxobacteria, we identified several strains producing branched, dialkylated pyrazines. *Chondromyces crocatus* (strains Cm c2 and Cm c5) and *Nannocystis exedens* (Na c29) both release **2** among other pyrazines. Furthermore, small amounts of the regioisomeric 2,6-diisopropylpyrazine (**3**) are produced by *C. crocatus* [17–19].

We became interested in the biosynthesis of **2** and **3**, because their amino acid dependence pointed towards a different mechanism compared to the biosynthesis of the highly alkylated pyrazines of the first class. Since these compounds, in contrast to the amino acid-derived pyrazines from the third class, do not carry any O-containing functional groups, a different pathway compared to the formation of methoxypyrazines was expected. To shed more light on this biosynthetic pathway, differently labeled amino acid derivatives were synthesized and fed to the myxobacteria *N. exedens* and *C. crocatus*. These experiments resulted in the elucidation of a mechanism for the bacterial formation of branched dialkylpyrazines *via* highly reactive amino aldehyde intermediates that is presented in this study.

Results and Discussion. – Our initial suggestion for the biosynthesis of **2** was based on the proposal by *Gallois et al.* [12] and *Cheng et al.* [13] (*Scheme 3*), starting with self-condensation of the amino acid valine (**10**) to the piperazinedione **16**. Compared to the proposed biosynthetic pathway to methoxypyrazines (*Scheme 2*), an additional reduction step is needed to remove the excess O-atom. Reduction of the C=O group would furnish the hemiaminal **18** which, upon elimination of H_2O, would give rise to the dihydro-hydroxy-pyrazine **19**. Rearrangement and H_2O elimination would furnish the target compound **2**. The formation of its isomer **3** would require a rearrangement of the C-backbone by an unknown mechanism.

To test this hypothesis, several feeding experiments with isotopically labeled precursors and proposed intermediates of this pathway were carried out. These labeled compounds were subjected to agar plate cultures of the bacteria, and their incorporation into the pyrazines **2** and **3** was monitored using a closed-loop stripping apparatus (CLSA) to collect the volatiles [17–19], followed by analysis of the

Scheme 3. *Initially Proposed Biosynthetic Pathway to 2,5-Diisopropylpyrazine* (**2**)



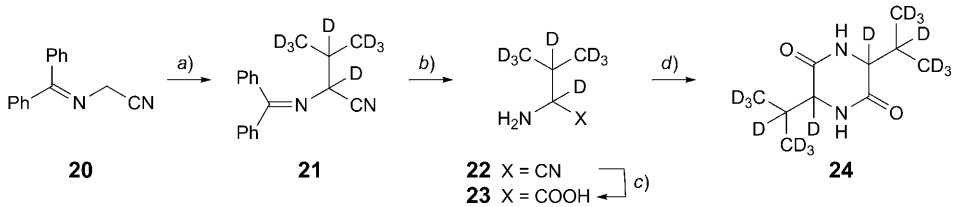
headspace extracts by GC/MS. The use of deuterium ($^2H_1$) labels was preferred because of the shorter GC retention times of deuterated analogs compared to the parent compounds [20]. This allowed the recording of almost clean mass spectra of deuterated isotopomers that would not be possible with ^{13}C or ^{15}N labels. The results were used to delineate the biosynthetic pathway.

Feeding Experiments with [$^2H_8$]Valine. Feeding of [$^2H_8$]valine to *C. crocatus* (Cm c5) resulted in the one- and twofold incorporation into pyrazine **2**. Predominantly, [$^2H_7$]-**2** with one labeled i-Pr group was found (incorporation rate 22%), but traces of [$^2H_{14}$]-**2** were also present (incorporation rate 2%). Similarly, [$^2H_8$]valine was incorporated into **3** with one or two labeled side chains, clearly establishing the formation of both pyrazines from two valine units. Feeding of an isotopomer of the piperazinedione **16** with two labeled side chains could demonstrate its intermediacy in the proposed pathway to **2** and could give additional insight into the pathway to **3**. Therefore, the extensively labeled piperazinedione **24** was synthesized as a mixture of both diastereoisomers and fed to the bacteria.

The synthesis of **24** started with the alkylation of 2-[(diphenylmethylidene)amino]acetonitrile (**20**) with 2-bromo[$^2H_7$]propane under basic conditions using NaOD in D_2O with simultaneous H/D exchange to yield **21** (*Scheme 4*). The diphenylmethylidene protecting group was cleaved off under acidic conditions, and the nitrile **22** was saponified to the carboxylic acid to yield *rac*-[$^2H_8$]valine (**23**) [21]. Its thermal dehydration [22] that has recently been used for the formation of leucine anhydride [23] resulted in a diastereoisomer mixture of **24**, albeit in low yield only.

Feeding Experiments with [$^2H_{16}$]Valine Anhydride (**24**). Previous work with the myxobacterium *N. exedens* (Na c29) showed that agar plate cultures of this bacterium produced **2** as one of the major volatiles [18]. Accordingly, this strain was used to probe whether **16** is an intermediate in the biosynthesis of **2**. Feeding of **24** to an agar plate culture as described in [18] clearly resulted in the incorporation of the isotopic labeling into **2** (*Figs. 1* and *2*), but the outcome was unexpected. Interestingly, besides unlabeled **2**, not only the expected [$^2H_{14}$]-**2** was detected, but also [$^2H_7$]-**2** was produced, corresponding to only one deuterated i-Pr group. The unlabeled 2,5-diisopropylpyrazine (**2**) shows a molecular-ion peak at *m*/*z* 164 and a base peak at *m*/*z* 149 that arises by loss of a Me group (*Fig. 2*). The corresponding ion peaks of [$^2H_7$]-**2** are shifted to *m*/*z*

Scheme 4. *Synthesis of [$^2H_{16}$]Valine Anhydride* (**24**)



a) $BnEt_3N^+Cl^-$, toluene, NaOD, 2-bromo[$^2H_7$]propane; 54%. *b*) Et_2O, 1M HCl. *c*) Conc. HCl, reflux; 83% (over two steps). *d*) SiO_2, Δ, 10%.

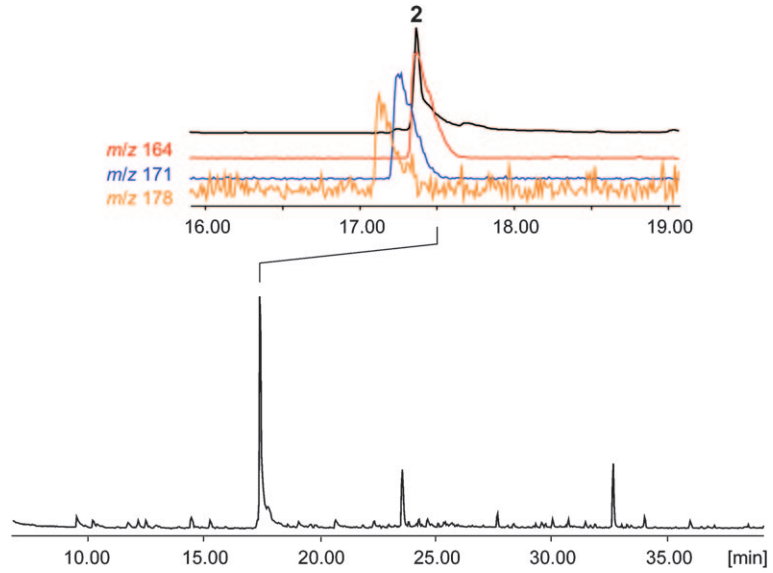


Fig. 1. *Total ion chromatogram of a headspace extract obtained after feeding of [$^2H_{16}$]valine anhydride* (**24**) *to* Nannocystis exedens (Na c29). The enlargement shows the ion traces of the molecular ion of isotopomers of **2**.

171, and 156 or 153, respectively, depending on the loss of a labeled or an unlabeled Me group. The compound [$^2H_{14}$]-**2** exhibits a molecular-ion peak at *m*/*z* 178 and a base peak at *m*/*z* 160 representing the loss of a CD_3 fragment from either of the labeled i-Pr groups. These mass spectral data together with the slightly decreased retention times of the deuterated compounds compared to those of unlabeled **2** unambiguously led to the identification of the labeled pyrazines.

Compound **24** was also fed to *C. crocatus* (Cm c5) that is known for its ability to produce a large variety of pyrazines, including the regioisomer of **2**, 2,6-diisopropylpyrazine (**3**) [17][19]. The results were similar to those obtained with *N. exedens* (Na c29), showing unlabelled, 2,5-[$^2H_7$]-, and 2,5-[$^2H_{14}$]diisopropylpyrazine (**2**). Additionally, weak incorporation of **24** into **3**, a compound not produced by *N. exedens*, was observed (*Fig. 3*), but, due to the low production rate of this volatile, only [$^2H_7$]-**3** was

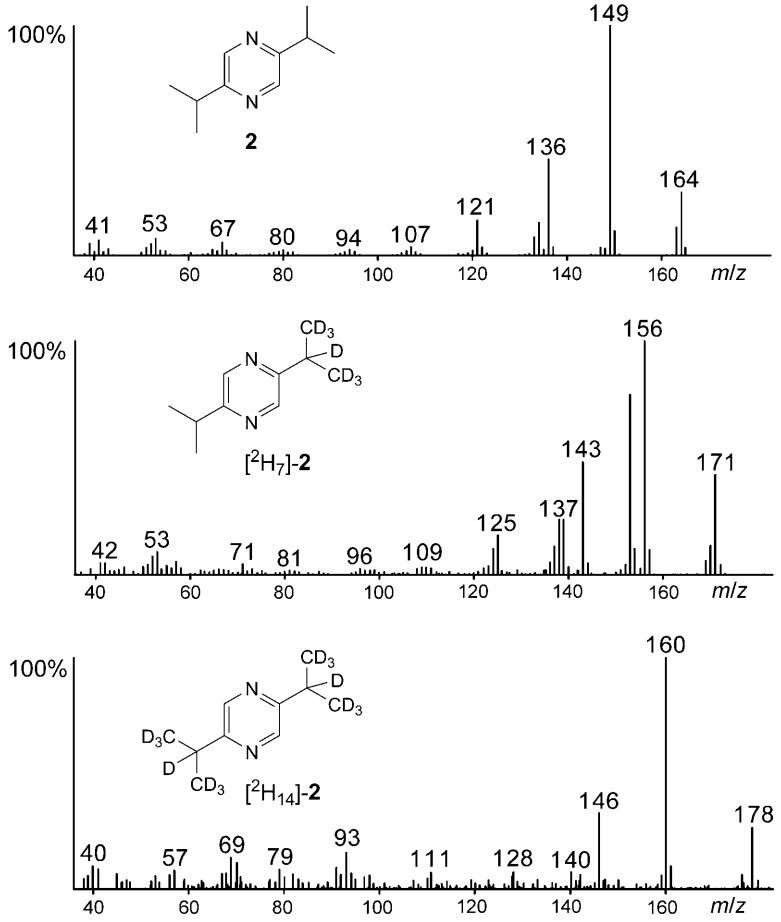


Fig. 2. *Mass spectra of a headspace extract obtained after feeding of [$^2H_{16}$]valine anhydride* (**24**) *to* Nannocystis exedens (Na c29)

identified by the characteristically shifted ion peaks at *m/z* 153, 156, and 171, while [$^2H_{14}$]-**3** was missing.

The detection of [$^2H_{14}$]-**2** is in accordance with the incorporation of the complete precursor **24**, whereas the formation of [$^2H_7$]-**2** requires hydrolysis of the cyclic [$^2H_{16}$]dipeptide **24** into two molecules of labeled valine. One labeled valine and another unlabeled valine could then be used to rebuild [$^2H_8$]-**16**, meaning that the production of [$^2H_7$]-**2** and [$^2H_7$]-**3** does not principally rule out the intermediacy of this piperazinedione in the biosynthesis of both pyrazines. However, the $^2H_1$-incorporation rates for *N. exedens* were 20% for [$^2H_7$]-**2** and 1% for [$^2H_{14}$]-**2**, and, similarly, for *C. crocatus* 30% for [$^2H_7$]-**2** and 5% for [$^2H_{14}$]-**2**, raising doubts on the intermediacy of **16**. If **16** would be a true intermediate on the pathway to **2** and **3**, and if a partial hydrolysis of [$^2H_{16}$]-**16** to [$^2H_8$]valine and back-formation of [$^2H_8$]-**16** would give rise to [$^2H_7$]-**2** and [$^2H_7$]-**3**, the incorporation rates for these pyrazines, derived from one labeled valine, would

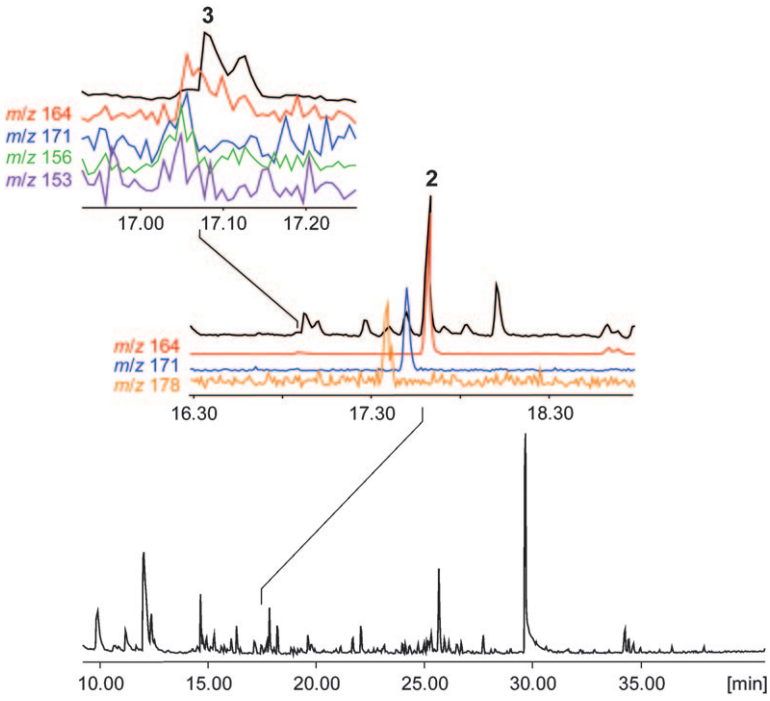


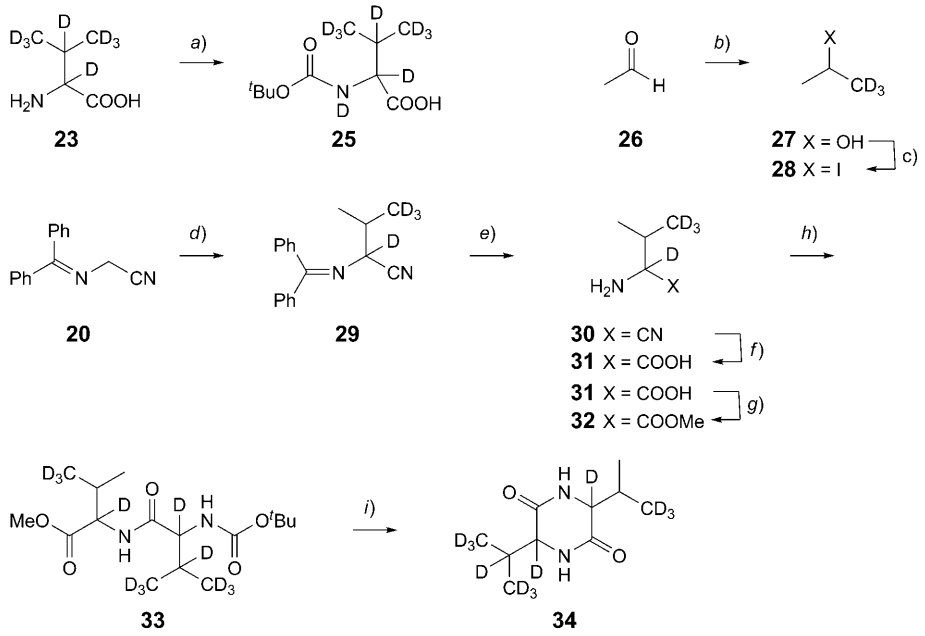
Fig. 3. *Total ion chromatogram of a headspace extract of* Chondromyces crocatus (Cm c5) *obtained after feeding of [$^2H_{16}$]valine anhydride* (**24**). The enlargement shows the ion traces of the molecular ion of isotopomers of **2** and **3**.

expectedly be lower than the incorporation rates into the isotopomers derived from two labeled valine units as present in the supplied **24**. It seems more likely that the bacteria completely cleave the cyclic dipeptide into two amino acid units, and then use the pool of labeled and unlabeled valine to produce the observed isotopomeric mixture of **2** on another pathway.

To differentiate between the two possibilities, labeled valine anhydride **34** containing differently labeled [$^2H_4$]valine and [$^2H_8$]valine units was synthesized and used in feeding experiments. If **16** is a true intermediate, [$^2H_{10}$]-**2** would be formed in higher proportions relative to other isotopomers, while its hydrolysis into valine and transformation *via* another pathway would lead to different isotopomers of **2** in low concentration. Therefore, [$^2H_{12}$]valine anhydride **34** was synthesized, containing seven and three $^2H_1$-atoms in the i-Pr side chains.

Synthesis of [$^2H_{12}$]Valine Anhydride **34**. Previously synthesized [$^2H_8$]valine (**23**) was protected with a Boc group to give **25** as one building block of the target compound [24] (*Scheme 5*). [2,4,4,4-$^2H_4$]Valine (**31**) was synthesized starting with a *Grignard* reaction of [$^2H_3$]methylmagnesium iodide with acetaldehyde (**26**), followed by iodination of the obtained [1,1,1-$^2H_3$]propan-2-ol (**27**) to 2-iodo[1,1,1-$^2H_3$]propane (**28**) [25] that was used in the alkylation of **20**. The resulting nirile **29** was transformed as described above for the synthesis of **23** into *rac*-[2,4,4,4-$^2H_4$]valine (**31**) [21] that was subsequently

Scheme 5. *Synthesis of [$^2H_{12}$]Valine Anhydride* (**34**)



a) NaOD, D_2O, Boc_2O, dioxane; 12%. *b*) [$^2H_3$]MeMgI, Et_2O. *c*) HI (57%), reflux. *d*) $BnEt_3N^+Cl^-$, toluene, NaOD, **28**; 37%. *e*) Et_2O, 1M HCl. *f*) Conc. HCl, reflux; 6% (over two steps). *g*) $SOCl_2$, MeOH; 31%. *h*) Boc-[$^2H_9$]Valine, ClCOO(i-Br), Et_3N, CH_2Cl_2. *h*) **25**, 1,2-Dichlorobenzene, reflux; 5% (over two steps).

protected as methyl ester **32**. Coupling of **25** and **32** formed dipeptide **33** [26][27] that underwent deprotection and cyclization to **34** in refluxing 1,2-dichlorobenzene [27].

Feeding Experiments with [$^2H_{12}$]Valine Anhydride **34**. Feeding of **34** to *N. exedens* resulted only in the production of [$^2H_3$]-**2** (molecular ion at *m*/*z* 167) originating from the hydrolysis product [$^2H_4$]valine, and [$^2H_7$]-**2** (*m*/*z* 171, from [$^2H_8$]valine) besides large amounts of unlabelled **2** (*Fig. 4*). The isotopomer [$^2H_{14}$]-**2** (*m*/*z* 178, from two units of [$^2H_8$]valine) was detected in trace amounts only. The concentration was near the limit of detection. The other expected isotopomers including [$^2H_{10}$]-**2** that could directly be formed from **34** as a hypothetical intermediate and [$^2H_{14}$]-**2** could not be verified; their concentration was most likely below the limit of detection. The relative ratios of **2**, [$^2H_3$]-**2**, [$^2H_7$]-**2**, [$^2H_6$]-**2**, [$^2H_{10}$]-**2**, and [$^2H_{14}$]-**2** are roughly 90 : 5 : 5 : 0.06 : 0.13 : 0.06, when one assumes statistical use of valine from a pool of valine, [$^2H_4$]valine, and [$^2H_8$]valine in a relative ratio of 95 : 2.5 : 2.5, showing the low probability of the formation of **2** from two labeled valine molecules. The relative ratio of **2**, [$^2H_3$]-**2**, and [$^2H_7$]-**2** actually found was *ca.* 95 : 3 : 2. The presence of only trace amounts of [$^2H_{14}$]-**2** in the previous experiment together with the absence of [$^2H_{10}$]-**2** in this experiment indicate that the labeled isotopomers of **16** are indeed hydrolyzed to yield two labeled valine molecules that serve as precursors for the biosynthesis of **2** in another pathway. A direct transformation of **16** into **2** can be ruled out.

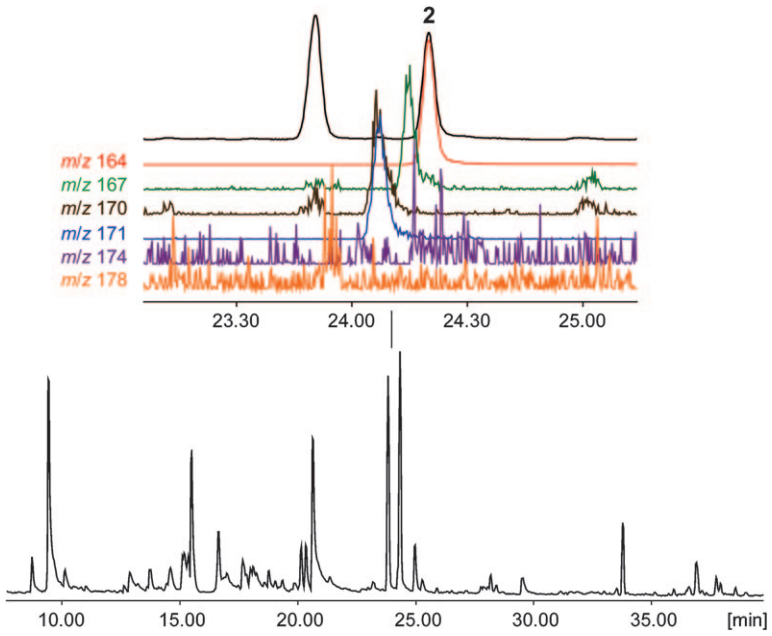


Fig. 4. *Total ion chromatogram of a headspace extract of* N. exedens (Na c29) *obtained after feeding of [$^2H_{12}$]valine anhydride* (**34**). The enlargement shows the ion traces of the molecular ion of isotopomers of **2**.

The feeding experiment with **34** was repeated with *C. crocatus* (Cm c2) that also produces both diisopropylpyrazines **2** and **3** [17], to see whether larger amounts of the compounds were produced, compared to strain Cm c5 (*Fig. 5*). In this experiment, **2**

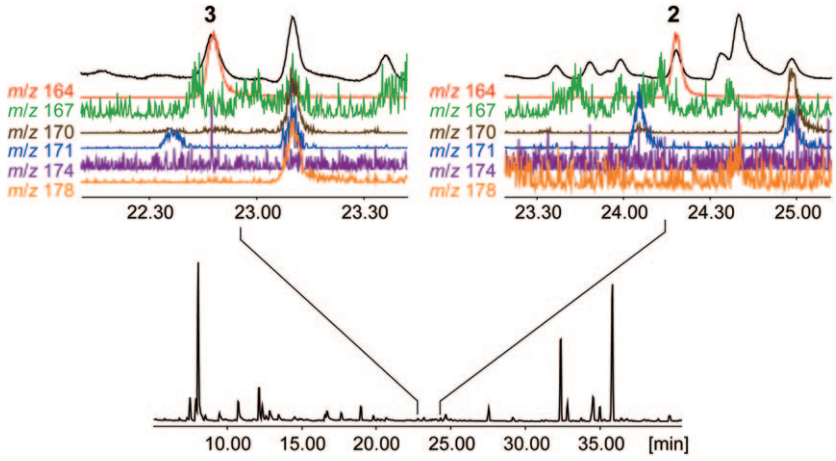


Fig. 5. *Total ion chromatogram of a headspace extract of* C. crocatus (Cm c2) *obtained after feeding of [$^2H_{12}$]valine anhydride* (**34**). The enlargements show the ion traces of the molecular ion of isotopomers of **2** and **3**.

and **3** with only one labeled i-Pr group were detected (molecular-ion peaks were observed either at *m/z* 167 or 171, resp.), whereas all three possible isotopomers derived from two labeled valine units were absent. These results similarly rule out the intermediacy of **16** for this species.

Feeding Experiments with [^{15}N]Valine. Upon addition of [^{15}N]valine to cultures of *C. crocatus* (Cm c2 and Cm c5), both strains showed an enhanced production of **2** and **3**. The one- and twofold incorporation of ^{15}N-labeling was indicated by enhanced intensities of the ions at *m/z* 165 and 166 compared to their natural abundance in the unlabeled compounds (*Fig. 6*). The relative amounts of **2**, [^{15}N]-**2**, and [$^{15}N_2$]-**2** were 55 : 35 : 10, calculated from the base peak at *m/z* 149, because the presence of an abundant $[M-1]^+$ ion in the mass spectrum of **2** made quantification by use of the molecular ion ambiguous. A similar ratio, 52 : 36 : 12, was found for **3**, [^{15}N]-**3**, and [$^{15}N_2$]-**3** (*Fig. 6*). The ratio is roughly equal to the calculated ratio of isotopomers (57 : 29 : 14) when one fifth of the valine used by the bacteria was labeled with ^{15}N. The results clearly established the origin of the N-atoms in the pyrazines from valine and excluded any participation of a transamination process during the biosynthesis of **2** and **3**.

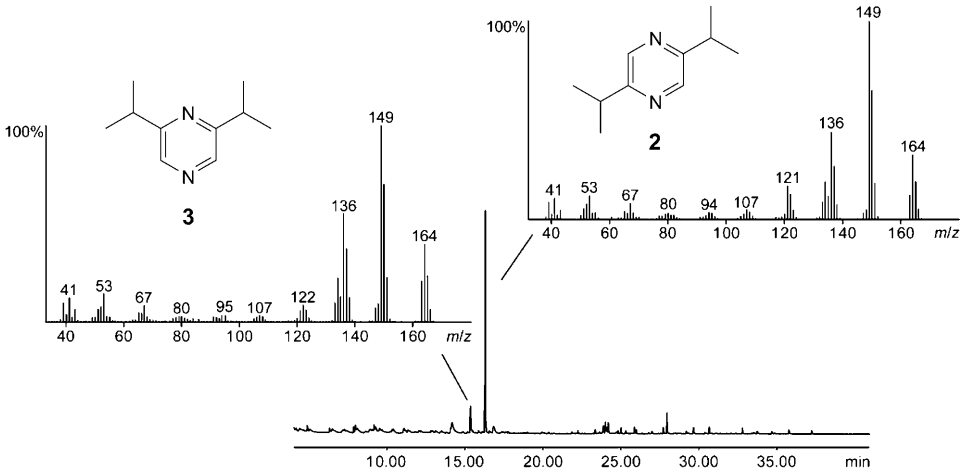


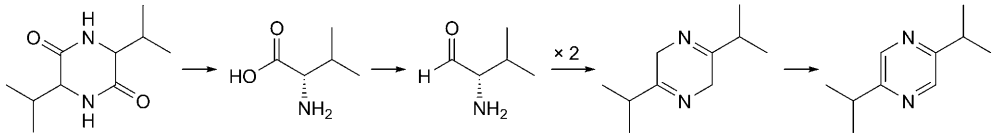
Fig. 6. *Total ion chromatogram and mass spectra of* **2** *and* **3** *in a headspace extract of* C. crocatus (Cm c5) *obtained after feeding of [^{15}N]valine*

The Biosynthetic Pathway to 2,5-Diisopropylpyrazine (**2**) *and Its Regioisomer* **3**. The results of the feeding experiments showed that the myxobacteria *N. exedens* and *C. crocatus* metabolize cyclic valine dipeptide by hydrolysis and not by direct modification. The piperazinedione serves merely as a source of the amino acid valine. No evidence for an equilibrium between the cyclic dipeptide and the free amino acid was found. Two units of valine are then combined to form the pyrazines **2** and **3**, as shown by the formation of [$^{2}H_{14}$]-**2** and [$^{15}N_2$]-**2** from [$^{2}H_8$]- and [^{15}N]valine, respectively. Since an excess of unlabeled valine originating from the medium or by *de novo* synthesis compared to labeled valine was available to the bacteria in the feeding experiments, the

formation of unlabeled or mono-labeled pyrazines was favored over the doubly-labeled compounds. These results rule out a piperazinedione intermediate in the biosynthesis of dialkylated pyrazines of the second class as proposed in *Scheme 2*.

Instead, a pathway *via* an unstable α-amino aldehyde intermediate seems more likely (*Scheme 6*), similar to the biosynthesis of alkylated pyrazines from acetoin and related α-hydroxycarbonyl compounds [7]. Valine (**10**) is reduced to the highly reactive α-amino-aldehyde intermediate **35** that can dimerize by two condensation reactions to form the dihydropyrazine **36**. Finally, **36** is oxidized enzymatically or nonenzymatically to form the target pyrazine **2**. The occurrence of dihydropyrazine intermediates in the biosynthesis of pyrazines is supported by the recent identification of 3,6-dihydro-2,5-dimethylpyrazine occurring besides 2,5-dimethylpyrazine in emissions of the fruit fly *Anastrepha serpentina* [28]. Pathways requiring a valine amide as intermediate [10] or a transamination step can be clearly ruled out because of the retention of the valine N-atom.

Scheme 6. *Proposed Biosynthetic Pathway to 2,5-Diisopropylpyrazines* (**2**)

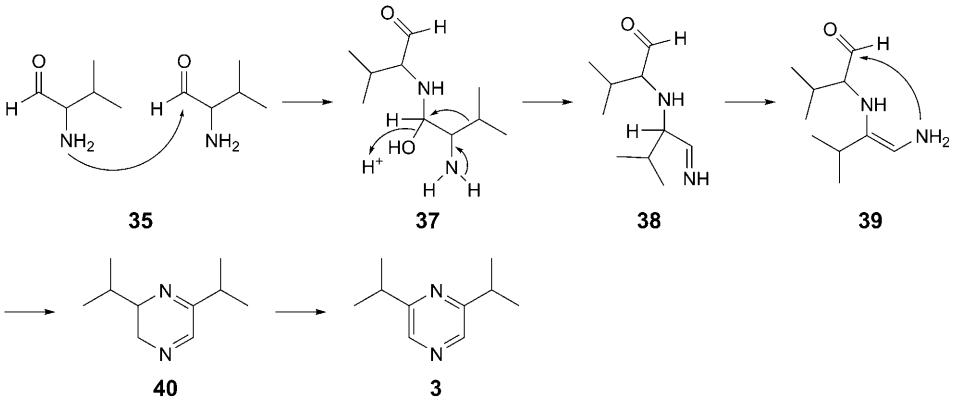


16 **10** **35** **36** **2**

The presence of small amounts of **3** might be explained by a rearrangement during the biosynthesis. α-Amino aldehydes are well-known to be autocatalytically transformed into more stable amino ketones [29][30]. Since in the chemical reaction two α-amino aldehydes react under exchange of their N-atoms, such a process seems unlikely under physiological conditions. Instead, the isomerization would require an external nitrogen source, possibly delivered by a transamination process. During this sequence, the N-atom of the α-amino aldehyde would be lost and replaced by an N-atom from an external source. Therefore, the ^{15}N-content of the isomer [$^{15}N_2$]-**3**, obtained in the feeding experiments with [$^{15}N_2$]valine, should be significantly lower than that of [$^{15}N_2$]-**2**. This is not the case, indicating the absence of a 1-amino-3-methylbutan-2-one intermediate in the biosynthesis of **3**.

Instead, a rearrangement during the condensation of two α-amino-aldehyde units **35** as shown in *Scheme 7* seems more plausible and is in accord with the observed ^{15}N-labeling pattern. The attack of the NH_2 group of **35** on the C=O group of another molecule **35** leads to the tetraedric intermediate **37** that conventionally undergoes elimination of H_2O to form an imine. An identical reaction on the other carbonyl group leads to compound **36**, and eventually to **2**. In the biosynthesis of **3**, however, H_2O is eliminated, and the i-Pr group is rearranged to the former C=O C-atom [31]. The reaction commences with H^+ abstraction from the second N-atom, leading to the primary imine **38**. Tautomerization of the imine to the enamine furnishes the ene-diamine **39** that condenses to the dihydropyrazine **40**. The final oxidation furnishes the rearranged pyrazine **3**, without requirement for an external N-atom. Therefore, the ^{15}N-labeling patterns in compounds **2** and **3** remain identical.

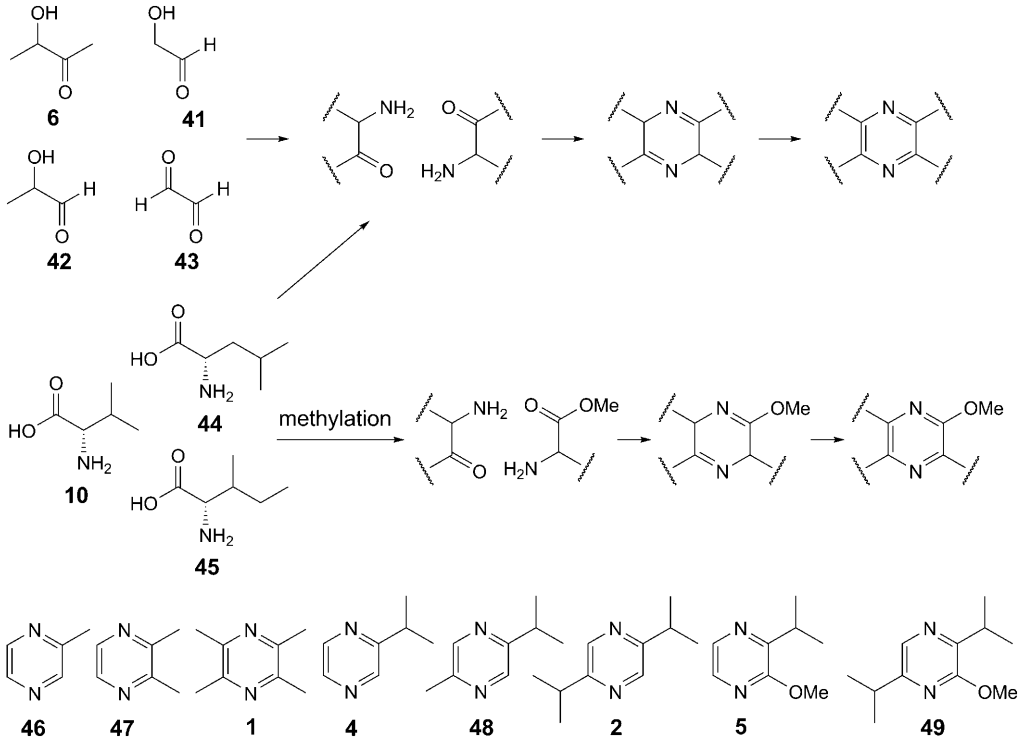
Scheme 7. *Proposed Biosynthetic Pathway to 2,6-Diisopropylpyrazines* (**3**)



The proposed biosynthetic pathway may also be operative for other 2,5- and 2,6-dialkylpyrazines, starting from the amino acids leucine (**44**), isoleucine (**45**), or others. It explains also why no 2,3-dialkylpyrazines are formed, since the structure of the precursor does not allow their formation. Interestingly, many of the educts of the pyrazine biosynthesis such as acetoin (**6**) [7] or the highly reactive α-amino aldehyde **35** are toxic to bacteria. It seems reasonable that some bacteria use the formation of the pyrazines as a detoxification mechanism to get rid of these compounds.

The biosynthetic pathway proposed here is similar to the pathway to alkylated pyrazines of class one [7]. While the amino acids **10**, **44**, and **45** are used as starting materials in the formation of branched dialkylpyrazines, the unbranched alkylated pyrazines are formed *via* α-hydroxycarbonyl compounds such as acetoin (**6**), glycolaldehyde (**41**), lactaldehyde (**42**), or probably glyoxal (**43**). The α-aminocarbonyl compounds derived from these building blocks may be combined as shown in *Scheme 8*, leading to a multitude of product pyrazines represented by the compounds **1**, **2**, **4**, **5**, and **46**–**49**. α-Amino aldehydes are chemically unstable and may dimerize even without enzymatic activity to form dihydropyrazines that either undergo disproportionation or are oxidized, *e.g.*, by air, to form pyrazines [11][32][33]. Nevertheless, it seems likely that the dimerization process is enzyme-controlled because distinct pyrazine patterns occur in different bacteria. Up to now, no enzyme responsible for the dimerization or the following oxidation step is known. Finally, also the formation of methoxypyrazines of type three can be explained by the described pathway. If one α-aminocarbonyl unit in the condensation is replaced by an amino acid methyl ester, a dihydromethoxypyrazine would be formed that could be finally oxidized to the methoxypyrazine. The *O*-Me group could also be added in later stages if the initial condensation is performed by an amino acid instead of the methyl ester. The labeling studies by *Gallois et al.* [12] who observed incorporation of **10**, but not of glycine, and *Cheng et al.* [13] are in accord with the biosynthetic pathway proposed in *Scheme 8*. The earlier proposal by *Murray et al.* [10] suggesting the formation of methoxypyrazines by condensation of glyoxal with valine amide is a variant of our proposal and can be differentiated by ^{15}N-labeling studies.

Scheme 8. *General Biosynthetic Scheme for the Bacterial Formation of Pyrazines*



In summary, our results strongly point to reactive α-aminocarbonyl species as key intermediates in pyrazine biosynthesis. The results fit into a unifying pyrazine biosynthetic pathway, explaining the formation of three important pyrazine classes found in Nature.

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Experimental Part

General. Chemicals were purchased from *Sigma-Aldrich* (Germany), *Fluka* (Switzerland), *Acros* (Belgium), *Merck* (Germany), *CDN* (Canada) *via Dr. Ehrenstorfer* (Germany), or *CS* (Germany). All solvents were purified by distillation and dried according to standard methods. TLC: 0.2-mm pre-coated plastic sheets (*Polygram Sil G/UV$_{254}$*; *Macherey-Nagel*). Column chromatography (CC): *Merck* silica gel *60* (SiO_2; 70–200 mesh) and solvent mixtures as used for the determination of R_f values in TLC. Compounds were detected using UV light or a ninhydrin soln., followed by heat-gun treatment. NMR Spectra: *Bruker DPX-200*, *AVII-300*, *DRX-400*, or *AVII-600* spectrometers; δ in ppm rel. to Me_4Si as internal standard, J in Hz.

Media and Growth Conditions. The strains *Nannocystis exedens* Na c29, and *Chondromyces crocatus* Cm c2 and Cm c5 were grown on VY/2 agar plates (5 g l^{-1} baker yeast, 0.1 g l^{-1} $CaCl_2 \cdot 2\ H_2O$, 0.5 mg l^{-1}, vitamin B_{12}, 15 g l^{-1} agar, pH 7.2). The feeding experiments were performed in plastic *Petri* dishes

containing 25 ml of VY/2 agar. Strikes of well-grown agar cultures were each placed centrally on fresh agar plates, and the plates were incubated at 30° for 2 d (Cm c2) to 6 d (Cm c5 and Na c29) until approximately one third to half of the plates were covered with cells. Then, the labeled precursors (0.1 mM final concentration) dissolved in H_2O and filter-sterilized were striked out from the end of the grown cells on the surface area of the agar plates. The treated agar cultures were further incubated at 30° for 1–2 d (Cm c2 and Cm c5) and 7 d (Na c29), resp., and then analyzed.

Sampling of Volatiles. Volatile org. compounds emitted by liquid cultures of myxobacteria were collected using the CLSA technique [17]. The volatiles were adsorbed on charcoal (*Chromtech*; Precision Charcoal Filter, 5 mg) for 24 h and afterwards eluted with 30 µl of CH_2Cl_2. The solns. were immediately analyzed by GC/MS, and stored at −30°.

GC/EI-MS Analysis. GC/EI-MS Analyses were carried out on a *HP-6890* GC system connected to a *HP-5973* mass-selective detector fitted with a BPX5 fused-SiO_2 cap. column (25 m, 0.22 mm i.d., 0.25 µm film; SGE), or on an *Agilent 7890A* GC system connected to an *Agilent 5975C* inert mass detector fitted with a *HP-5MS* fused SiO_2 cap. column (30 m, 0.25 mm i.d., 0.25 µm film; *Agilent*). Conditions for the *HP-6890*/*HP-5973* system were as follows: inlet pressure, 77.1 kPa; 23.3 ml He min^{-1}; injection volume, 1 µl; transfer line, 300°; electron energy, 70 eV. Conditions for the *Agilent 7890A*/*Agilent 5975C* system were as follows: inlet pressure: 77.1 kPa, He 23.3 ml min^{-1}; injection volume: 2 µl; transfer line: 300°; electron energy: 70 eV. The GC was programmed as follows: 5 min at 50°, increasing at 5° min^{-1} to 320°, operated in either split or splitless mode (60-s valve time); He carrier gas at 1 ml min^{-1} (*HP-6890*) or 1.2 ml min^{-1} (*Agilent 7890A*). Retention indices (*I*) were determined from a homologous series of *n*-alkanes (C_8–C_{35}) [34]. Identification of compounds was performed by comparison of the mass spectra with those in the *Wiley-6 Library*, and by comparison with those of synthetic standards and of retention indices from the literature [17][18].

*Derivatization with 2,2,2-Trifluoro-*N*-methyl-*N*-(trimethylsilyl)acetamide* (MSTFA). Silylations were performed by adding MSTFA to the corresponding compound without the addition of any solvent as described in [35]. After 60 min at 60°, the silylated compounds were concentrated under a stream of N_2, dissolved in CH_2Cl_2, and then analyzed by GC/MS.

Preparation of 2-[(Diphenylmethylidene)amino]-3-methylbutanenitriles. The following compounds were prepared according to *Baldwin et al.* [21] either using commercially available 2-bromo[$^2H_7$]propane or 2-iodo[1,1,1-$^2H_3$]propane (**28**). 2-Iodo[1,1,1-$^2H_3$]propane (**28**) was synthesized as described by *Chaykovski et al.* [25] using [$^2H_3$]MeI as starting compound. The reaction progress was monitored by GC/MS. The crude product was used for the synthesis of **29**, because isolation of **28** proved difficult due to its high volatility.

2-Iodo[1,1,1-$^2H_3$]propane (**28**). EI-MS (70 eV): 173 (45, M^+), 158 (1), 155 (1), 127 (62), 46 (100).

2-[(Diphenylmethylidene)amino]-3-([$^2H_3$]methyl)[2,3,4,4,4-$^2H_5$]butyronitrile (**21**). Yield: 54% (591 mg, 2.20 mmol). R_f (pentane/Et_2O 10:1) 0.23. GC: *I* 2047. ^{1}H-NMR (400 MHz, $CDCl_3$): 7.13–7.82 (*m*, 10 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 17.6 (*m*, 2 [$^2H_3$]Me); 32.5 (*m*, C[$^2H_1$]); 59.0 (*m*, C[$^2H_1$]); 118.9 (C); 127.4 (2 CH); 128.2 (2 CH); 128.9 (2 CH); 129.2 (2 CH); 131.0 (2 CH); 135.4 (C); 138.6 (C); 172.8 (C). EI-MS: 270 (7, M^+), 252 (2), 220 (60), 219 (5), 180 (4), 165 (21), 117 (100), 77 (14), 51 (6).

2-[(Diphenylmethylidene)amino]-3-methyl[2,4,4,4-$^2H_5$]butanenitril (**29**). Yield: 37% (530 mg, 1.99 mmol). R_f (pentane/Et_2O 10:1) 0.20. GC: *I* 2051. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.01 (*d*, *J* = 6.8, 3 H); 1.12 (*d*, *J* = 6.8, 3 H); 2.11 (*q*, *J* = 6.6, 2 H); 7.18–7.82 (*m*, 20 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 18.4 (*m*, Me, [$^2H_3$]Me); 18.8 (*m*, Me, [$^2H_3$]Me); 33.3 (2 CH); 118.9 (2 C); 127.5 (4 CH); 128.2 (4 CH); 129.0 (4 CH); 129.2 (4 CH); 131.0 (4 CH); 135.5 (2 C); 138.7 (2 C); 172.9 (2 C). EI-MS: 266 (3, M^+), 251 (1), 220 (44), 180 (10), 165 (30), 117 (100), 77 (26), 51 (16), 46 (15).

Preparation of Valine Isotopomers. The different valine isotopomers were prepared according to the procedure of *Baldwin et al.* [21] by stirring either nitrile **21** or **29** (1 equiv.) for 12 h at r.t. in a mixture of Et_2O (8 ml) and 1M HCl (16 ml). Then, the org. layer was removed, and the aq. layer was extracted twice with Et_2O. Conc. HCl (16 ml) was added to the aq. layer, and the resulting soln. was heated to reflux for 12 h. Concentration of the product under vacuum gave a crude product that was dissolved in H_2O and subjected to ion-exchange CC (*DOWEX 1 × 8*, 200–400 mesh, Cl^-). Concentration *in vacuo* furnished the product.

[2,3,4,4,4,4′,4′,4′-$^2H_8$]Valine (**23**). Yield: 83% (600 mg, 4.80 mmol). GC: *I* 1182 (Bis(trimethylsilyl) (TMS) adduct). ^{13}C-NMR (100 MHz, D_2O): 18.2 (*m*, [$^2H_3$]Me); 19.7 (*m*, [$^2H_3$]Me); 30.9 (*m*, C[$^2H_1$]); 62.7 (*m*, C[$^2H_1$]); 177.1 (C). EI-MS (Bis-TMS adduct): 254 (3, $[M-15]^+$), 226 (11), 219 (21), 164 (2), 133 (2), 119 (2), 101 (6), 73 (58), 60 (4), 45 (7).

[2,4,4,4-$^2H_4$]Valine (**31**). Yield: 6% (17 mg, 0.14 mmol). GC: *I* 1186 (Bis-TMS adduct). ^{1}H-NMR (300 MHz, D_2O): 1.06 (*d*, *J* = 6.8, 3 H); 1.08 (*d*, *J* = 6.8, 3 H); 2.24 (*q*, *J* = 6.8, 2 H). ^{13}C-NMR (100 MHz, D_2O): 18.9 (*m*, Me, [$^2H_3$]Me), 21.0 (*m*, Me, [$^2H_3$]Me); 33.0 (CH), 56.3 (*m*, C[$^2H_1$]), 179.2 (C). EI-MS (Bis-TMS adduct): 250 (3, $[M-15]^+$), 223 (3), 222 (11), 160 (2), 148 (100), 132 (5), 101 (6), 73 (61), 59 (3), 45 (8).

Preparation of [$^2H_{16}$]Valine Anhydride (**24**). As described by *Basiuk* [22], [$^2H_8$]valine (85 mg, 0.7 mmol) was mixed in a sublimation apparatus with 400 mg of SiO_2 which was previously dried at 250° in a high vacuum. The mixture was then heated to 230° under high vacuum conditions for 45 min, upon which the product (16 mg, 0.07 mmol) sublimed. The product was obtained in a diastereoisomeric mixture (ratio 2 : 1) with a yield of 10%.

Data of **24**. GC: *I* 1332/1358. GC: *I* 1332/1358. ^{1}H-NMR (400 MHz, $CDCl_3$): 3.89 (*d*, *J* = 1.7, 1 H, H/D exchange); 3.94 (*d*, *J* = 1.2, 1 H, H/D exchange); 5.90–5.94 (*m*, 4 H). ^{13}C-NMR (100 MHz, D_2O): 19.0 (*m*, 8 [$^2H_3$]Me); 31.1 (*m*, 4 C[$^2H_1$]); 61.2 (*m*, CH, C[$^2H_1$]); 62.2 (*m*, CH, C[$^2H_1$]); 169.3 (2 C); 172.7 (2 C). EI-MS (70 eV) (*I* 1332): 213 (2, $[M-H]^+$), 194 (4), 165 (100), 142 (12), 135 (25), 115 (60), 107 (13), 88 (20), 80 (45), 73 (11), 61 (19), 46 (25)./(*I* 1358): 214 (1, M^+), 194 (3), 164 (100), 141 (19), 135 (21), 123 (15), 115 (49), 106 (11), 86 (20), 79 (68), 61 (31), 46 (31).

Preparation of N-*[(*tert-*Butoxy)carbonyl][*N,*2,3,4,4,4,4′,4′,4′-$^2H_9$]valine* (**25**). According to the procedure of *Bayardon* and *Sinou* [24], the Boc protecting group was introduced using NaOD in D_2O instead of NaOH to avoid any H/D exchange during the reaction. Aq. workup gave 50 mg (0.22 mmol) of the pure product, containing a D-atom also bonded to N-atom.

Data of **25**. Yield: 12%. GC: *I* 1489. ^{1}H-NMR (400 MHz, $CDCl_3$): 1.45 (*s*, 9 H); 9.80 (br. *s*, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 16.5 (*m*, [$^2H_3$]Me); 17.9 (*m*, [$^2H_3$]Me); 28.2 (3 Me); 30.0 (*m*, C[$^2H_1$]); 57.9 (*m*, C[$^2H_1$]); 80.0 (C); 155.8 (C); 177.0 (C). EI-MS: 226 (1, M^+), 180 (17), 152 (3), 124 (54), 105 (5), 80 (54), 57 (100), (41).

Preparation of [2,4,4,4-$^2H_4$]Valin Methyl Ester (**32**). This compound was prepared by using the procedure of *Herbert* and *Knaggs* [26] for the synthesis of amino acid methyl ester hydrochlorides. The obtained methyl [$^2H_4$]valinate hydrochloride was then isolated using ion-exchange chromatography (*DOWEX 1* × *8*, 200–400 mesh, Cl^-) giving 95 mg (0.70 mmol) of the product containing some side product.

Data of **32**. Yield: 31%. GC: *I* 971 (MSTFA). ^{1}H-NMR (400 MHz, CD_3OD): 1.02–1.09 (*m*, 6 H); 2.21 (*q*, *J* = 6.7, 2 H); 3.84 (*s*, 6 H). ^{13}C-NMR (100 MHz, CD_3OD): 17.5 (*m*, Me, [$^2H_3$]Me); 18.5 (*m*, Me, [$^2H_3$]Me); 30.4 (CH); 30.6 (CH); 53.3 (Me); 53.4 (Me); 59.1 (*m*, 2 C[$^2H_1$]); 170.6 (C); 171.5 (C). EI-MS (MSTFA): 207 (1, M^+), 192 (4), 161 (62), 148 (100), 132 (7), 101 (6), 89 (14), 73 (70), 59 (8), 45 (7).

Preparation of N-*[(*tert-*Butoxy)carbonyl)[$^2H_{12}$]divaline Methyl Ester* **33**. As described by *Chen et al.* [27], the two molecules **25** and **32** were coupled, and the obtained crude product was used for the next reaction without further purification.

Data of **33**. GC: *I* 1904/*I* 1939. EI-MS (*I* 1904): 342 (1, M^+), 281 (4), 255 (2), 239 (13), 207 (22), 192 (10), 180 (8), 166 (4), 124 (54), 80 (100), 57 (61), 41 (20). EI-MS (*I* 1939): 342 (1, M^+), 286 (2), 269 (6), 255 (2), 209 (9), 180 (20), 152 (2), 134 (9), 124 (86), 102 (5), 80 (100), 76 (20), 57 (60), 41 (9).

Preparation of [$^2H_{12}$]Valine Anhydride **34**. According to the procedure of *Chen et al.* [27], this compound was prepared by heating **33** for 18 h at 180° in 1,2-dichlorobenzene. The mixture was then cooled to 50°, MTBE was added cautiously, and then further cooled to r.t. The solid formed during the reaction was centrifuged off, washed several times with CH_2Cl_2, dissolved in MeOH, and treated with charcoal, which was filtered off afterwards. Removal of the solvent under vacuum finally gave the pure diastereoisomeric product (ratio 2 : 1).

Data of **34**. Yield: 5% (4 mg, 0.02 mmol; over two steps). GC: *I* 1752/*I* 1778. ^{1}H-NMR (600 MHz, CD_3OD): 0.82–0.85 (*m*, 12 H); 0.94 (*d*, *J* = 7.2, 3 H); 0.94 (*d*, *J* = 7.2, 3 H); 0.96 (*d*, *J* = 7.2, 3 H); 0.96 (*d*, *J* = 7.2, 3 H); 2.16 (*q*, *J* = 6.9, 4 H); 2.21 (*q*, *J* = 7.1, 4 H). ^{13}C-NMR (150 MHz, CD_3OD): 16.6 (2 Me); 17.6 (*m*, 24 [$^2H_3$]Me); 17.8 (2 Me); 18.6 (2 Me); 19.2 (2 Me); 30.9 (*m*, 4 C[$^2H_1$]); 33.0 (*m*, 4 CH, 4 C[$^2H_1$]); 33.4 (*m*,

4 CH); 60.9 (*m*, 16 C[$^2H_1$]); 170.3 (8 C); 170.6 (8 C). EI-MS (*I* 1752): 210 (1, M^+), 192 (2), 165 (100), 136 (17), 116 (85), 104 (8), 88 (29), 80 (36), 76 (19), 58 (20), 50 (11), 46 (20). EI-MS (*I* 1778): 210 (1, M^+), 192 (3), 165 (100), 139 (22), 115 (77), 107 (7), 87 (32), 80 (58), 76 (40), 58 (25), 50 (11), 46 (23).

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