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Facile Access to Isotopically Labelled Valylleucyl Anilides as Biomarkers for the Quantification of Hemoglobin Adducts to Toxic Electrophiles

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An easy and efficient synthesis of valylleucyl anilide (HVal-LeuNHPh) and labelled HVal($^{13}C_5$,^{15}N)LeuNHPh has been developed. Derivatization of these substances with oxirane, acrylonitrile, epichlorohydrin, glyceraldehyde and other aldehydes gives a series of reference substances and internal standards for the quantitative evaluation of human exposure to toxic electrophiles by quantitative determination of their hemoglobin adducts. Coupling of *N*-Z-*N*-Me-Val($^{13}C_5$,^{15}N)OH with HLeuNHPh followed by hydrogenolysis affords *N*–Me–Val($^{13}C_5$,^{15}N)LeuNHPh for quantification of the exposure to methylating agents.

Introduction

Human exposure to toxic electrophilic substances may be monitored by measuring hemoglobin (Hb) adducts with these compounds or their reactive metabolites.[1] In blood, electrophilic toxicants may attack all possible nucleophilic centers of Hb. All four chains of human Hb have the same *N*-terminal valine residue. Therefore, detection and quantitative determination of *N*-modified valine termini in Hb may serve as a cumulative index for human exposure to alkylating agents, Michael acceptors (acrylonitrile) and other electrophilic pollutants. Classical Edman degradation[2] has been used as a simple and sensitive procedure for cleaving off and analyzing *N*-terminal amino acid residues in proteins. Modified terminal *N*-alkylated valine residues in Hb were shown to be released easily in the reaction with phenyl or perfluorophenyl isothiocyanate.[1a,1g,3] Spontaneous cyclization and cleavage of the corresponding *N*-alkyl-(perfluoro)phenylthiohydantoins under basic conditions followed by their extractive separation led to the development of the so-called "*N*-alkyl" Edman procedure for GC-MS quantification of Hb adducts.[4] This very helpful method has been adopted in many laboratories around the world and recommended for official use in Germany.[5]

However, up to now there was no uniform set of reference substances and internal standards for this method. For this purpose, *N*-modified valine derivatives which mimic the whole Hb molecule have been used.[6] To combine a reference substance and an internal standard in one chemical entity, the method of isotopic dilution is often applied for quantification. For example, a Swedish group reported the chemical transformations of radioactively labelled (^{3}H and ^{14}C) and deuterated samples of epichlorohydrin followed by their conjugation with globin to prepare standard globins.[1g] A similar methodology with sulfur mustard-[D_8] and [^{35}S] sulfur mustard has also been reported by another group.[4d] Incorporation of a (radioactive) label into the "variable" part of the molecule attached to the terminal valine residue is inconvenient for monitoring various toxic electrophiles, and therefore this approach has not been pursued any further. For routine use, it would be much better to develop an analytical procedure in which isotopic dilution with stable isotopes incorporated into the valine residue is adopted. Farmer et al. had reported about monitoring the exposure to acrylonitrile. Their quantification was based on *N*-(2-*c*yanoethyl)-[D_8]Val-Leu-Ser-OH which may be synthesized from the commercially available [D_8]valine, but a synthetic procedure was not published.[4g,4h]

To establish a new reference set, we incorporated another commercially available labelled L-valine [HVal($^{13}C_5$,^{15}N)OH][7] into the "classical" HValLeuNHPh standards.[8] Labelling with ^{13}C and ^{15}N is advantageous, because it al-

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ters physical properties of the standards (GC and HPLC retention times) much less than (per)deuteration, thus reducing the risk that the labelled standard will give a separate peak in GC or HPLC traces and complicate the calibration procedure when applying the isotopic dilution technique. In addition, D-labelled substances tend to exchange D vs. H, especially in acidic or alkaline media which are often used in the extraction procedures prior to instrumental analysis.

Here we report general procedures for the (reductive) alkylation and Michael addition of HVal($^{13}C_5$,^{15}N)LeuNHPh which may find wide use. Some unlabelled compounds of this type are commercially available,[9] but their synthesis has never been published. The easy production and derivatization of labelled and unlabelled HValNHPh reported here opens a toolbox for preparing a new series of internal standards for various biomonitoring procedures.

Results and Discussion

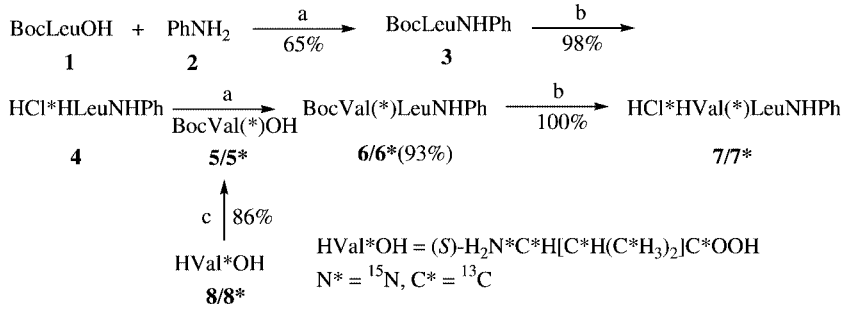
The labelled and unlabelled dipeptide precursors **7*/7** were prepared according to the sequence summarized in Scheme 1.[10]

Coupling of BocLeuOH with 7-aza-1-hydroxybenzotriazole (HOAt) followed by the reaction with aniline (**2**) gave the amide **3**. Deprotection of **3** with a solution of HCl in EtOAc produced the amine **4** as a hydrochloride, and subsequent amidation of the (labelled) BocVal*OH (**5/5***)[11] with **4** afforded the intermediate **6/6*** in excellent yield. Deprotection of the amino group in **6/6*** resulted in the final dipeptide **7/7***, which was isolated as the hydrochloride as well, and it was used for further transformations as such. All reactions, except the first one with inexpensive BocLeuOH and aniline, were found to be high yielding. To further reduce the costs of reagents, it is possible to perform this coupling of **1** and **2** with inexpensive *N*-hydroxybenzotriazole (HOBt) or *N*-hydroxysuccinimide, instead of the more expensive HOAt. In this case the yield was only insignificantly lower (60 vs. 65%).

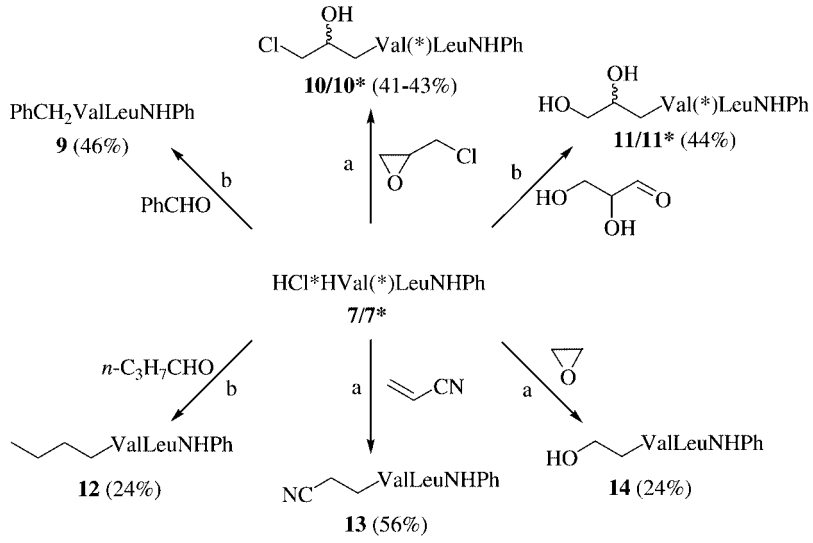
Reactions of **7/7*** with various alkylating agents were used to mimic the corresponding transformations of the *N*-valine terminus in hemoglobin (Scheme 2). Epichlorohydrin (ECH) which is an important monomer for the production of epoxide resins, as an alkylating agent has been classified as a category 2 carcinogen and allergen.[12] Alkylation of **7** (as a free base) with ECH leads to the compound **10** which mimics the initially formed Hb adduct of ECH. However, another modified Hb was found in vivo after exposure to ECH. It is represented by compound **11** with a hydroxy group instead of a chlorine atom. A plausible explanation for this fact is that in vivo, ECH also metabolizes to 3-chloro-1,2-propanediol or glycidol. All three compounds may react with the terminal valine residue of Hb, and their further transformations may end up with the single stable product *N*-(2,3-dihydroxypropyl)valine.[1g] For a quantification of ECH exposure, in addition to compound **10/10***, the diol **11/11*** was synthesized. Towards that, the reductive alkylation of **7/7*** with racemic glyceraldehyde was applied,[13] to give a mixture of two diastereomers in 49% yield. Similarly, reductive alkylations of **7/7*** with benzaldehyde and butanal afforded the corresponding model compounds **9** and **12** as mimics of the alkylation products of Hb by benzyl and butyl halides.

In these reductive alkylations, sodium (triacetoxy)-borohydride in dichloroethane consistently gave better results than $Na(CN)BH_3$ in methanol. However, attempted reductive alkylations of **7** with formaldehyde and acetaldehyde each gave inseparable mixtures of the starting compound, *N*-monoalkyl and *N*,*N*-dialkyl derivatives. Similar complications in the reductive alkylations with formaldehyde[6] and other aldehydes are known.[14] Nevertheless, reductive alkylation is a much more reliable method for the preparation of the analytical standards modelling the alkylated Hb termini. Alkyl and benzyl halides do not react selectively enough, and always produce mixtures of *N*-alkyl- and *N*,*N*-dialkylvalines, as well as *N*,*N*,*N*-trialkylammonium salts.

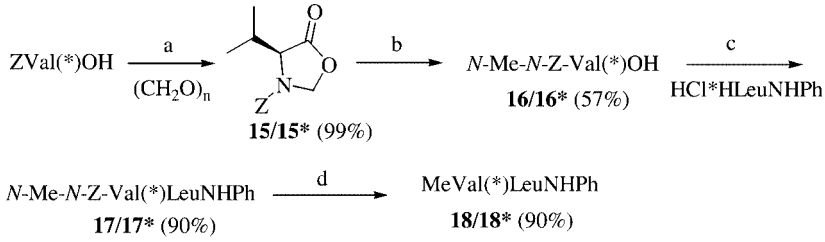
Another very strong alkylating agent, oxirane (ethylene oxide), produces the important reference compound **14**. The very toxic and reactive monomer acrylonitrile, readily reacts with **7** and by way of a Michael addition gives *N*-(2-cyanoethyl)valine **13**. Preparative yields of the reference substances in Scheme 2 are far from being quantitative. Our goal was to get pure substances, and inevitable losses during



Scheme 1. Synthesis of the dipeptide precursor(s) **7/7***: a) 7-aza-1-hydroxybenzotriazole (HOAt), *N*-(3-dimethylamino)propyl-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl), iPr_2NEt, CH_2Cl_2; b) 4 M HCl in EtOAc, CH_2Cl_2; c) di-*tert*-butyl pyrocarbonate (Boc_2O), *t*BuOH, aq. NaOH, 16 h, room temp.



Scheme 2. Synthesis of (labelled) analytical standards from HCl·HVal(*)LeuNHPh: a) iPr$_2$NEt, EtOH, room temp. 3 d; b) water/1,2-dichloroethane, 4-Å molecular sieves, Et$_3$N, then NaBH(OAc)$_3$, 35 °C, 2–5 h.



Scheme 3. Synthesis of MeVal(*)LeuNHPh via oxazolidinone **16**: a) camphor-10-sulfonic acid (CSA), toluene, reflux, 30 min; b) Et$_3$SiH, TFA, CHCl$_3$, room temp., 3 d; c) EDC·HCl, iPr$_2$NEt, CH$_2$Cl$_2$, HOBt, DMAP, room temp., overnight; d) H$_2$, 10% Pd/C, EtOH.

the purification to some extent explain the low isolated yields which refer to thoroughly purified materials obtained after column chromatography and recrystallization.

Another important reference is compound **18/18*** with the *N*-methylated valine terminus. It may be used for the evaluation of human exposure to hazardous methylating agents such as diazomethane, iodomethane and dimethyl sulfate. Direct synthesis of MeValLeuSerOH from HValLeuSerOH[15] by reductive alkylation with formaldehyde and Na(CN)BH$_3$ has been reported to be inconvenient, because it was necessary to apply "semipreparative" HPLC for the separation of the monomethylated product from by-products and an unknown impurity which was present in the starting material.[6] Therefore, another route was followed, along which *N*-Me–*N*-Z–ValOH (**16**) was first prepared in two steps from ZValOH via (*S*)-3-Z-4-isopropyloxazolidin-5-one (**15**) applying a known protocol (Scheme 3).[16] Coupling of *N*-Me–*N*-Z–ValOH with HCl·HLeuNHPh followed by hydrogenolysis produces MeValLeuNHPh in high overall yield and purity. The same route was used for the synthesis of the labelled internal standard MeValLeuNHPh.

The constitution and purity of the unlabelled final reference compounds were confirmed by elemental analyses. Predictably, this method did not work for the labelled standards, if, after combustion, the contents of carbon dioxide and nitrogen were measured by physical methods, in which the calibrations were made with unlabelled carbon dioxide and nitrogen. For example, measuring thermal conductivities of carbon dioxide and nitrogen during their GC determination or IR intensities of the preselected CO_2 and N_2 bands consistently gave values of carbon and nitrogen contents which were too low. Therefore, labelled standards were characterized by mass and NMR spectrometry.

Conclusions

An easy and efficient synthesis of HValLeuNHPh and labelled HVal($^{13}C_5$,^{15}N)LeuNHPh along with exemplified procedures of their (reductive) alkylation with aldehydes and their nucleophilic addition to reactive Michael acceptors has made a variety of internal standards and reference substances easily available. Analytical chemists engaged in determinations of hemoglobin adducts to toxic substances using the method of isotopic dilution may apply this protocol in their own work and synthesize various new reference sets depending on the nature of a hazardous electrophile. Successful application of the compound **11** in a highly sensitive analytical procedure for the determination of epichlorohydrin exposure has already been described.[17]

Experimental Section

General Remarks: Melting points (uncorrected) were determined in capillaries using a Büchi 510 apparatus. Routine NMR spectra were recorded with Varian MERCURY-300 and MERCURY-200 spectrometers at 300 (^{1}H) and 75.5 MHz (^{13}C and APT), as well as at 200 (^{1}H) and 50.3 MHz (^{13}C and APT), respectively. All spectra are referenced to tetramethylsilane as an internal standard (δ = 0 ppm) using the signals of the residual protons of deuterated solvents: 7.26 for $CHCl_3$, 2.50 for [D_5]DMSO and 3.30 for [D_3]-MeOH. Multiplicities of signals are described as follows: s = singlet, d = doublet, t = triplet, q = quadruplet, quint = quintuplet, sept = septuplet, m_c = centrosymmetrical multiplet. Coupling constants (J) are given in Hz. EI-MS were recorded with MAT 95 (70 eV) and ESI-MS with LCQ spectrometers (Fa. Finnigan). IR: Bruker IFS 66 (FT-IR) spectrophotometer, measured as KBr pellets or oils between KBr plates. Analytical TLC was performed on Macherey–Nagel ready-to-use plates AluGram Sil G/UV_{254}; detection with UV light (254 nm), development with molybdatophosphoric acid solution (5% in EtOH) or 0.5% aq. $KMnO_4$. Column chromatography: Merck silica gel, grade 60, 230–400 mesh. Elemental analyses were carried out at the Mikroanalytisches Laboratorium des Instituts für Organische und Biomolekulare Chemie der Georg-August-University of Göttingen. Solvents were purified according to standard procedures. Organic solutions were dried over $MgSO_4$. All reactions were carried out with magnetic stirring, unless stated otherwise.

The procedures used for the preparation of the compounds **3**, **4**, **6**, **6***, **7**, **7***, **17**, **18**, **18*** are given in the Supporting Information to this article and published electronically (for details see also the footnote on the first page of this article).

Benzyl (*S*)-4-Isopropyl-5-oxooxazolidine-3-carboxylate (15/15*)[18] **and *N*-benzyloxycarbonyl-*N*-methyl-L-valine (*N*-Z-*N*-MeValOH, 16/16*)**[19] were synthesized starting from ZValOH (Fluka) or ZVal*OH[20] according to a previously reported general method[6] (Scheme 3).

***N*-Benzylvalylleucyl Anilide (9):** To a solution of compound **7** (342 mg, 1.00 mmol) and *i*Pr_2Net (129 mg, 1.00 mmol) in 1,2-dichloroethane (DCE, 20 mL), was added molecular sieves (4 Å, 15 g) followed by freshly distilled benzaldehyde (212 mg, 2.00 mmol). After stirring for 30 min at room temperature, $NaBH(OAc)_3$ (424 mg, 2.00 mmol) was added, and the mixture was stirred at room temperature overnight. Then it was filtered, the molecular sieves were washed several times with dichloromethane, the combined filtrates (200 mL) were washed once with saturated aq. solution of $NaHCO_3$ (100 mL) and dried. After evaporation of the solvent in vacuo, the residue was separated by column chromatography on silica gel (50 g) eluting with chloroform/methanol (50:1) to afford **9** (180 mg, 46%), m.p. 131–133 °C. ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.93 (d, J = 6.9 Hz, 3 H), 0.96 (d, J = 6.8, 3 H), 0.99 (br. d, J = 6.8, 6 H), 1.59–1.76 (m, 3 H, CH_2 and CH in Leu), 1.89 (m_c, J = 6.9, 1 H, C*H*Me_2 in Val), 2.91 (d, J = 6.2, 1 H, C*H*NH_2 in Val), 3.59 (d, J = 12.5, 1 H, PhC*H*H), 3.79 (d, J = 13.1, 1 H, PhCH*H*), 4.63 (dd, J = 5.0 and 9.4 Hz, 1 H, C*H*NH in Leu), 7.09 (tm, J = 7.5 and 1.2 Hz, 1 H, 4-H_{Ar}), 7.17–7.34 (m, 7 H), 7.29 (tm, J = 7.5, 2 H, 3-H_{Ar}), 7.54 (dm, J = 7.5, 2 H, 2-H_{Ar}). ^{13}C NMR (75.5 MHz, [D_4]MeOH, ppm): δ = 19.2, 19.8, 22.0, 23.4, 26.1, 32.9, 42.3 (CH_2), 53.3 (CH_2), 53.4 (CH), 68.4 (CH), 121.4 (CH), 125.4 (CH), 128.1 (CH), 129.4 (CH), 129.5 (CH), 129.8 (CH), 139.6 (C_{ipso}), 141.0 (C_{ipso}), 172.9 (CO), 176.6 (CO). MS (ESI, positive mode): m/z (%) = 813 (100) [2M + Na^+], 791 (27) [2M + H^+], 418 (25) [M + Na^+], 396 (19) [M + H^+], negative mode: 440 (87) [M + HCO_2^-], 394 (100) [M – H^+]. IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3278, 3145, 3064, 2958, 2871, 1666, 1641, 1606, 1551, 1501, 1468, 1444, 1385, 1367, 1311, 1249, 1157, 1028. $C_{24}H_{33}N_3O_2 \cdot 0.25H_2O$ (400.04): calcd. C 72.09.56, H 8.38, N 10.51; found C 72.22, H 8.39, N 10.42.

(2*R*/*S*)-*N*-(3-Chloro-2-hydroxypropyl)valylleucyl Anilide (10): To a solution of compound **7** (342 mg, 1.00 mmol) and *i*Pr_2Net (129 mg, 1.00 mmol) in EtOH (10 mL), was added epichlorohydrin (92.5 mg, 1.00 mmol), and the reaction mixture was left to stand for 3 days. After concentration in vacuo, the residue was separated by chromatography on silica gel (50 g), eluting with CH_2Cl_2/EtOH/conc. aq. NH_3 (250:5:1) to afford **10** (163 mg, 41%) as a mixture of 2 diastereomers (1:1); m.p. 95 °C. ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.9–1.0 (m, 12 H), 1.59–1.76 (m, 3 H, CH_2 and CH in Leu), 1.94 (m_c, J = 6.9, 1 H, C*H*Me_2 in Val), 2.58–2.65 (m, J_{AB} = 13, 1 H, C*H*HN), 2.72–2.79 (m, J_{AB} = 13, 1 H, CH*H*N), 2.93 (t, J = 7.5 Hz, 1 H, C*H*NH_2 in Val), 3.58 (m, 2 H, $ClCH_2$), 3.84 (m, 1 H, C*H*OH), 4.61 (m, 1 H, C*H*NH in Leu), 7.09 (t, J = 7.5 Hz, 1 H, 4-H_{Ar}), 7.24 (t, J = 7.5 Hz, 2 H, 3-H_{Ar}), 7.56 (d, J = 7.5 Hz, 2 H, 3-H_{Ar}). ^{13}C NMR (75.5 MHz, [D_4]MeOH, ppm): δ = 19.9, 19.0, 19.7, 21.8, 21.9, 23.5, 26.1, 32.7, 42.2 (CH_2), 42.3 (CH_2), 52.6 (CH_2), 52.8 (CH_2), 53.4 (CH), 53.5 (CH), 69.6 (CH), 70.1 (CH), 71.6 (CH), 121.5 (CH), 125.4 (CH), 129.8 (CH), 139.5 (C_{ipso}), 141.0 (C_{ipso}), 173.1 (CO), 176.3 (CO). MS (CI, NH_3): m/z (%) = 400 (19) and 398 (62) [M + H^+], 242 (100), 209 (69). IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3290, 2959, 1643, 1603, 1548, 1500, 1468, 1445, 1388, 1310, 1249, 1197, 1075. $C_{20}H_{32}N_3O_3Cl$ (397.93): calcd. C 60.38, H 8.05, N 10.57; found C 60.65, H 7.90, N 10.51.

(2*R*/*S*)-*N*-(3-Chloro-2-hydroxypropyl)valyl($^{13}C_5$,^{15}N)leucyl Anilide (10*): The title product was obtained similarly to the unlabelled compound **10** and isolated in 43% yield; m.p. 95 °C. ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.97 (dm, 6 H, J = 126, $^{13}CH_3$ in Val), 0.99 (d×2, J = 6.5, 6 H, Me in Leu), 1.59–1.79 (m, 3 H, CH_2 and CH in Leu), 1.96 (dm, J = 125, 1 H, ^{13}C*H*Me_2 in Val), 2.62 (m, J_{AB} = 13, 1 H, C*H*HN), 2.74 (m, J_{AB} = 13, 1 H, CH*H*N), 2.91 (dm, J = 125, ^{13}C*H*NH_2 in Val), 3.58 (m, 2 H, $ClCH_2$), 3.84 (m, 1 H, C*H*OH), 4.61 (m, 1 H, C*H*NH in Leu), 7.09 (t, J = 7.5 Hz, 1 H, 4-H_{Ar}), 7.24 (t, J = 7.5 Hz, 2 H, 3-H_{Ar}), 7.56 (d, J = 7.5 Hz, 2 H, 3-H_{Ar}). ^{13}C NMR (75.5 MHz, [D_4]MeOH, only chemical shifts of the labelled atoms are given, ppm): δ = 18.9, 19.1 (d×2, J = 36), 19.8 (br. d, J = 35), 32.8 (q, J = 36), 69.9 (m), 176.6 (d, J = 50). MS (ESI, positive mode): m/z (%) = 829 (100) [2M + Na^+], 426 (27) [M + Na^+]; negative mode: 448 (100) [M + 2Na – H^+].

(2*R*/*S*)-*N*-(2,3-Dihydroxypropyl)valylleucyl Anilide (11): To a solution of compound **7** (365 mg, 1.07 mmol) in water (5 mL) was added racemic dimer of glyceraldehyde (Fluka, 340 mg, 3.78 mmol) followed by 1,2-dichloroethane (DCE, 100 mL), Et_3N (0.142 mL, 1.00 mmol) and molecular sieves (4 Å, 17 g). The round-bottomed reaction flask was rotated on a rotary evaporator (without vacuum) for 2 h, then $NaBH(OAc)_3$ (1.20 g, 5.66 mmol) was added in one portion, and rotation was continued at 35 °C (bath temp.) for 2 h. TLC of the reaction mixture indicated complete consumption of the starting material and two new poorly resolved spots with lower R_f values (CH_2Cl_2/MeOH/conc. aq. NH_3, 100:7:1). The reaction mixture was filtered through a fritted glass filter, and the filter cake was washed with several portions of DCE (until the product spots could not be detected any more on the TLC plate). The combined organic solutions (ca. 350 mL) were washed with sat. aq. $NaHCO_3$ and then with brine (100 mL each). After drying and removal of the solvent in vacuo, the oily residue was subjected to chromatography on silica gel (50 g) eluting with CH_2Cl_2/MeOH/conc. aq. NH_3, 100:7:1. Compound **11** (R_f = 0.2) was isolated as an oil (0.22 g) which crystallized from a $CHCl_3$/hexane mixture to give 0.18 g (44%) of a colorless solid (1:1 mixture of 2 diastereomers).

The diastereomer with the higher R_f value was isolated from the head fraction; its ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.96 d (J = 6.8), 0.97 d (J = 6.0), 0.98 d (J = 7.5), 0.99 d (J = 6.0) (Σ, 12 H), 1.60–1.78 (m, 3 H, CH_2 and CH in Leu), 1.99 (m_c, J = 6.4, 1 H, C*H*Me$_2$ in Val), 2.58 (dd, J_{AB} = 12, J_{AX} = 6.7, 1 H, C*H*HN), 2.69 (dd, J_{AB} = 12, J_{AX} = 3.8, 1 H, CH*H*N), 2.89 (d, J = 6.4 Hz, 1 H, CHNH in Val), 3.54 (d, J = 6.0 Hz, 2 H, CH_2OH), 3.72 (m_c, 1 H, C*H*OH), 4.60 (dd, J = 4.5 and 10, 1 H, C*H*NH in Leu), 7.08 (tt, J = 7.5 and 1, 1 H, 4-H_{Ar}), 7.30 (tm, J = 7.8, 2 H, 3-H_{Ar}), 7.54 (dm, J = 7.8, 2 H, 3-H_{Ar}). ^{1}H NMR (300 MHz, $CDCl_3$+CD_3OD, mixture of 2 diastereomers, ppm): δ = 0.73–0.82 (Σ, 12 H, 4×Me), 1.48 (m, 3 H, CH_2 and CH in Leu), 1.86 (m, 1 H, C*H*Me$_2$ in Val), 2.36 (dd, J_{AB} = 12.5, J_{AX} = 6.6, C*H*HN), 2.45 (m), 2.56 (dd, J_{AB} = 12.4, J_{AX} = 3.4, Σ 2 H, CH*H*N), 2.69 and 2.72 (d×2, J = 5, Σ 1 H, CHNH in Val), 3.31–3.48 (m, 2 H, CH_2OH), 3.55 (m_c, 1 H, C*H*OH), 4.20 (m, 1 H, C*H*NH in Leu), 6.91 (t, J = 7.5 Hz, 1 H, 4-H_{Ar}), 7.11(t, J = 7.4 Hz, 2 H, 3-H_{Ar}), 7.33 (d, J = 7.4 Hz, 2 H, 3-H_{Ar}). ^{13}C NMR (75.5 MHz, $CDCl_3$+CD_3OD, mixture of 2 diastereomers, ppm): δ = 17.4, 17.6, 18.9, 21.0, 22.5, 24.5, 31.0, 40.9 (CH_2), 50.8 (CH_2), 51.52 (CH_2), 51.56 (CH), 64.1 (CH_2), 64.3 (CH_2), 68.2 (CH), 68.5 (CH), 70.4 (CH), 70.5 (CH), 120.0 (CH), 124.19 (CH), 128.5 (CH), 137.4 (C_{ipso}), 171.4 (CO), 174.7 (CO), 174.8. MS (CI, NH_3): m/z (%) = 380 (100) [M + H$^+$], 226 (10), 209 (22). $C_{20}H_{33}N_3O_4$ (379.50): calcd. C 63.30, H 8.76, N 11.07; found C 63.08, H 9.02, N 11.04.

(2*RlS*)-*N*-(2,3-Dihydroxypropyl)valyl($^{13}C_5$,^{15}N)leucyl Anilide (11*): The title product was obtained as described for the unlabelled compound **11** and isolated in 41% yield. ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.96 (dm, J = 128, 6 H, $^{13}CH_3$ in Val), 0.97 and 0.99 (d×2, J = 5.5, 6 H, Me in Leu), 1.62 and 1.71 (m, 3 H, CH_2 and CH in Leu), 1.96 (dm, J = 120, 1 H, ^{13}C*H*Me$_2$ in Val), 2.49–2.75 (m, 2 H), 2.92 (dm, J = 120, ^{13}C*H*NH$_2$ in Val), 3.57 (m, 2 H, ClCH$_2$), 3.67 (m, 1 H, C*H*OH), 4.60 (m, 1 H, C*H*NH in Leu), 7.08 (t, J = 7.5 Hz, 1 H, 4-H_{Ar}), 7.29 (t, J = 7.5 Hz, 2 H, 3-H_{Ar}), 7.53 (d, J = 8.2 Hz, 2 H, 3-H_{Ar}). ^{13}C NMR (75.5 MHz, [D$_4$]MeOH, only chemical shifts of the labelled atoms are given, ppm): δ = 18.8, 19.0 (d×2, J = 35), 19.8 (d, J = 35), 32.7 (q, J = 35), 69.9 (m), 176.7 (d, J = 51). MS (ESI, positive mode): m/z (%) = 793 (100) [2M + Na$^+$], 771 (19) [2M + H$^+$], 408 (8) [M + Na$^+$], 386 (18) [M + H$^+$]; negative mode: 430 (19) [M + 2Na – H$^+$], 384 (100) [M – H$^+$].

***N*-Butylvalylleucyl Anilide (12):** The title compound was prepared from 1 mmol of **7**, 3 mmol of freshly distilled butanal and 3 mmol of NaBH(OAc)$_3$ according to the method described above for compound **9**, yield 86 mg (24%), m.p. 135–137 °C. ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.9–1.0 (m, 15 H), 1.38 (m_c, 2 H), 1.48 (m_c, 2 H), 1.60–1.78 (m, 3 H, CH_2 and CH in Leu), 1.82 (m_c, J = 6.4, 1 H, C*H*Me$_2$ in Val), 2.53 (m, 2 H, CH$_2$N), 2.92 (d, J = 8 Hz, 1 H, CHNH in Val), 4.62 (dd, J = 5 and 10 Hz, 1 H, C*H*NH in Leu), 7.09 (tm, J = 7.5 and 1, 1 H, 4-H_{Ar}), 7.28 (tm, J = 7.8, 2 H, 3-H_{Ar}), 7.55 (dm, J = 7.8, 2 H, 3-H_{Ar}). ^{13}C NMR (75.5 MHz, [D$_4$] MeOH, ppm): δ = 14.3, 19.4, 19.6, 21.5 (CH_2), 22.0, 23.5, 26.1, 32.8, 33.0 (CH_2), 42.2 (CH_2), 52.6 (CH_2), 53.4 (CH), 69.4 (CH), 121.3 (CH), 125.4 (CH), 129.8 (CH), 139.6 (C_{ipso}), 172.8 (CO). MS (ESI, positive mode): m/z (%) = 745 (73) [2M + Na$^+$], 362 (100) [M + H$^+$]; negative mode: 360 (100) [M – H$^+$]. IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3271, 3146, 3093, 2957, 2931, 2871, 1665, 1640, 1608, 1553, 1501, 1467, 1444, 1386, 1312, 1250, 1179. $C_{21}H_{35}N_3O_2$ (361.52): calcd. C 69.81, H 9.70, N 11.63; found C 69.87, H 9.93, N 11.54.

***N*-(2-Cyanoethyl)valylleucyl Anilide (13):** The title compound was synthesized from equivalent amounts of **7** and acrylonitrile in the presence of *i*Pr$_2$Net (1 equiv.) in EtOH as described for product **10**, yield 56%, m.p. 145 °C. ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.9–1.1 (m, 12 H), 1.58–1.80 (m, 3 H, CH_2 and CH in Leu), 1.90 (m_c, J = 6.4, 1 H, C*H*Me$_2$ in Val), 2.59 (t, J_{AX} = J_{BX} = 8, 2 H, X-part of the ABX$_2$-system, CH$_2$CN/CH$_2$NH), 2.78 (A-part of the ABX$_2$-system, J_{AB} = 13, C*H*HNH/C*H*HCN), 2.83 (B-part of the ABX$_2$-system, J_{AB} = 13, CH*H*CN/CH*H*NH), 2.93 (d, J = 8 Hz, 1 H, CHNH in Val), 4.58 (dd, J = 5 and 10 Hz, 1 H, C*H*NH in Leu), 7.09 (t, J = 7.5 and 1, 1 H, 4-H_{Ar}), 7.28 (t, J = 7.8 Hz, 2 H, 3-H_{Ar}), 7.55 (d, J = 7.8 Hz, 2 H, 3-H_{Ar}). ^{13}C NMR (75.5 MHz, [D$_4$]-MeOH, ppm): δ = 18.9, 19.1 (CH_2), 19.8, 21.8, 23.5, 26.2, 32.7, 42.0 (CH_2), 45.2 (CH_2), 53.5 (CH), 69.2 (CH), 120.4 (CN), 121.4 (CH), 125.3 (CH), 129.8 (CH), 139.6 (C_{ipso}), 172.9 (CO), 176.5 (CO). MS (EI): m/z (%) = 358 (5) [M$^{+\cdot}$], 315 (8) [M$^+$ – C_3H_7], 224 (9), 191 (8), 125 (100). IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3274, 3204, 3146, 3094, 2957, 2929, 2872, 2851, 2247, 1667, 1637, 1607, 1552, 1494, 1468, 1443, 1386, 1368, 1313, 1277, 1243, 1178, 1157, 1109, 1081. $C_{20}H_{30}N_4O_2$ (358.48): calcd. C 67.04, H 8.38, N 15.64; found C 66.99, H 8.41, N 15.58.

***N*-(2-Hydroxyethyl)valylleucyl Anilide (14):** This compound was synthesized from **7** (342 mg, 1.00 mmol) and oxirane (44 mg, 1.00 mmol, 237 mg of 18.6% solution in THF) in the presence of *i*Pr$_2$Net (1 mmol) in EtOH (10 mL) as described for the product **10**, yield 84 mg (24%), m.p. 119 °C. ^{1}H NMR (300 MHz, CD_3OD, ppm): δ = 0.9–1.0 (m, 12 H), 1.59–1.79 (m, 3 H, CH_2 and CH in Leu), 1.96 (m_c, J = 6.4, 1 H, C*H*Me$_2$ in Val), 2.63 (m_c, 2 H, CH$_2$NH), 2.91 (d, J = 5.6 Hz, 1 H, CHNH in Val), 3.64 (t, J = 5.6 Hz, 2 H, CH$_2$OH), 4.60 (dd, J = 5 and 10 Hz, 1 H, C*H*NH in Leu), 7.08 (tt, J = 7.4 and 1, 1 H, 4-H_{Ar}), 7.29 (t, J = 8.1 Hz, 2 H, 3-H_{Ar}), 7.54 (d, J = 8 Hz, 2 H, 3-H_{Ar}). ^{13}C NMR (75.5 MHz, [D$_4$]-MeOH, ppm): δ = 18.9, 19.7, 19.8, 21.9, 23.5, 26.1, 32.7, 42.3 (CH_2), 51.8 (CH_2), 53.5 (CH), 62.2 (OCH_2), 69.7 (CH), 121.4 (CH), 125.4 (CH), 129.8 (CH), 139.6 (C_{ipso}), 173.2 (CO), 176.7 (CO). MS (ESI, positive mode): m/z (%) = 771 (100) [2M + Na$^+$], 699 (100) [2M + H$^+$], 350 (28) [M + H$^+$]; negative mode: 394 (100) [M + HCOO$^-$], 348(48) [M – H$^+$]. IR (KBr, cm^{-1}): $\tilde{\nu}$ = 3298, 3144, 3089, 2960, 2871, 1668, 1642, 1606, 1551, 1501, 1468, 1444, 1387, 1368, 1313, 1176, 1155, 1059. $C_{19}H_{31}N_3O_3$ (349.47): calcd. C 65.33, H 8.88, N 12.03; found C 65.10, H 8.60, N 11.82.

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