Synthesis of a psilocin hapten and a protein–hapten conjugate

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Abstract

Derivatives of psilocin with ω-functionalized alkyl spacers in position 1 of the indole ring were synthesized as haptens for use in a radioimmunoassay. Whereas the psilocin analogues with a 3-aminopropyl and a 4-aminobutyl moiety at the indole nitrogen decomposed during synthesis, the analogous 3-carboxypropyl psilocin derivative proved to be stable. This compound was coupled to bovine serum albumin (BSA) using the N-hydroxysuccinimide ester-mediated conjugation. The protein–hapten conjugate was characterized by matrix-assisted laser desorption ionization mass spectrometry. The mass spectrometry data indicated an average incorporation ratio of 4–5 molecules of psilocin hapten per molecule of BSA.

Introduction

The indole derivatives psilocybin and psilocin (Figure 1) were first identified as constituents of hallucinogenic mushrooms of the genus *Psilocybe* (Hofmann et al 1958; Hofmann & Troxler 1959). Later, these indoles were also found in a variety of other species of fungi (Stijve & Kuyper 1985; Gartz 1986; Semerdzieva et al 1986). Although psilocybin is the major component in comparison with psilocin in mushrooms, its phosphoric ester group is cleaved rapidly and extensively after ingestion of the mushrooms (Horita & Weber 1961, 1962; Kalberer et al 1962). These findings indicate that psilocybin acts as a prodrug and that its metabolite psilocin represents the pharmacologically active agent (Hasler et al 1997).

The abuse of psilocybin-containing hallucinogenic fungi has become an increasing problem in recent years (Musshoff et al 2000). Several methods have been described for the detection of psilocybin and psilocin in fungi, for example thin layer chromatography (Beug & Bigwood 1981), high-performance liquid chromatography (HPLC) with UV (Beug & Bigwood 1981; Sottolano & Lurie 1983; Musshoff et al 2000), fluorescence (Christiansen et al 1981) or electrochemical detection (Wurst et al 1992), gas chromatography-mass spectrometry (GC-MS) (Wurst et al 1992; Keller et al 1999) and capillary zone electrophoresis (Pedersen-Bjergaard et al 1997). Current methods for the determination of psilocin in body fluids are REMEDi HS as a drug screening method (Sticht & Käferstein 2000), HPLC with electrochemical detection (Kysilka 1990; Hasler et al 1997; Lindenblatt et al 1998), GC-MS (Sticht & Käferstein 2000; Grieshaber et al 2001) and liquid chromatography electrospray ionization mass spectrometry (Bogusz 2000). Whereas the determination of psilocin in urine can be performed without a sample clean up (Kysilka 1990), its analysis in plasma requires an extraction procedure followed by derivatization of the extracts. In forensic toxicology analyses, an immunoassay is often used (Carter et al 2000; De Letter et al 2001), because it offers the opportunity to detect a drug of abuse in plasma or urine sensitively without pre-treatment of the samples. However, such a method has not yet been published for psilocin.

Here, we describe the synthesis of a psilocin hapten and its coupling to bovine serum albumin (BSA). This BSA–hapten conjugate can be used for the development of a radioimmunoassay for psilocin.

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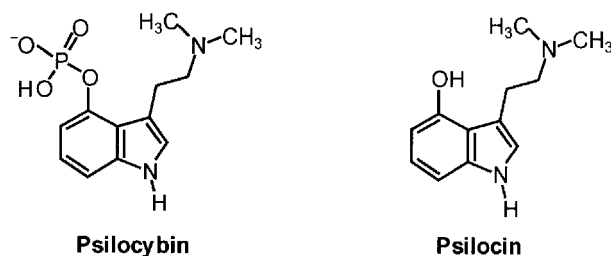
Figure 1   Structures of psilocybin and psilocin.

Materials and Methods

General

N-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDAC) polymer was purchased from Fluka (Taufkirchen, Germany). BSA was a gift from Dade Behring (Marburg, Germany). Silica gel chromatography was performed using silica gel 60 (70–230 mesh) from Merck (Darmstadt, Germany); ^{1}H NMR spectra were obtained on a Varian Gemini 200 NMR spectrometer. Chemical shifts are reported in δ units (ppm) relative to tetramethylsilane. The electron beam ionization (EI) mass spectra were obtained on a Thermo Finnigan GCQ (San Jose, USA). The high resolution mass spectrum applying electrospray ionization (HR-ESI-MS) was recorded on a Micromass Quattro-LC (Manchester, UK).

Synthesis of the psilocin hapten

Dibenzyl-{3-[4-benzyloxy-3-(2-dimethylaminoethyl)indol-1-yl]propyl}amine (**2**)

NaH (25 mg, 0.62 mmol) was dissolved in dry dimethylformamide (DMF; 8 mL) at 0°C and stirred for 30 min at this temperature. The solution of 4-benzyloxy-*N*,*N*-dimethyltryptamine (Yamada et al 1998; Nichols & Frescas 1999) (62 mg, 0.21 mmol) in dry DMF (14 mL) was added dropwise and the mixture was stirred for further 30 min at 0°C. The solution of dibenzyl(3-bromopropyl)amine (Nagle et al 2000) (165 mg, 0.52 mmol) dissolved in dry DMF (10 mL) was then added in the same way. The resulting mixture was stirred for 30 min in an ice bath, then for 4 h at 60°C, and subsequently overnight at room temperature. The mixture was diluted with 5% aqueous NaHCO$_3$ solution and extracted with diethyl ether. The organic phase was dried over MgSO$_4$ and evaporated. Silica gel chromatography with dichloromethane/methanol (1:1) produced **2** as a yellow waxy solid (83 mg, 74%). ^{1}H NMR (CDCl$_3$): 1.94 (quint, J = 7 Hz, 2H), 2.13 (s, 6H), 2.44–2.56 (m, 4H), 2.92–3.00 (m, 2H), 3.57 (s, 4H), 4.00 (t, J = 7 Hz, 2H), 5.18 (s, 2H), 6.43 (s, 1H), 6.51 (d, J = 8 Hz, 1H), 6.84 (d, J = 8 Hz, 1H), 7.04 (t, J = 8 Hz, 1H), 7.27–7.53 (m, 15H); MS (EI) m/z: 531.2 (M$^+$).

4-[4-Benzyloxy-3-(2-dimethylaminoethyl)indol-1-yl]butyronitrile (**4**)

NaH (119 mg, 3.0 mmol) was added to dry DMF (8 mL) at 0°C and stirred for 30 min. The solution of 4-benzyloxy-

N,*N*-dimethyltryptamine (398 mg, 1.53 mmol) in dry DMF (14 mL) was then added dropwise. After stirring the mixture for 30 min at 0°C, 4-bromobutyronitrile (228 mg, 1.53 mmol) dissolved in dry DMF (10 mL) was added in the same way. The mixture was stirred for an additional 30 min at the same temperature and overnight at room temperature. The mixture was diluted with 5% aqueous NaHCO$_3$ solution and extracted with diethyl ether. The organic phase was dried over MgSO$_4$ and the solvent evaporated. Silica gel chromatography with dichloromethane/ethyl acetate/triethyl amine (8:1:0.2, v/v/v) gave **4** as a waxy solid (237 mg, 49%). ^{1}H NMR (CDCl$_3$): 2.14 (s, 6H), 2.19–2.28 (m, 4H), 2.53–2.61 (m, 2H), 2.99–3.07 (m, 2H), 4.22 (t, J = 7 Hz, 2H), 5.19 (s, 2H), 6.56 (d, J = 8 Hz, 1H), 6.80 (s, 1H), 6,91 (d, J = 8 Hz, 1H), 7.10 (t, J = 8 Hz, 1H), 7.32–7.43 (m, 3H), 7.49–7.53 (m, 2H); MS (EI) m/z: 361.0 (M$^+$).

4-[4-Benzyloxy-3-(2-dimethylaminoethyl)indol-1-yl]butan-1-amine (**5**)

A solution of **4** (133 mg, 0.37 mmol) in methanol (10 mL) was added to Raney-Nickel (158 mg) in a hydrogenation flask. The mixture was stirred under a balloon filled with H$_2$ for 38 h at room temperature. The catalyst was removed by filtration through a glass filter. The solvent was evaporated to give **5** as a yellow waxy solid (116 mg, 86%). ^{1}H NMR (CDCl$_3$): 1.38–1.52 (m, 2H), 1.77–1.91 (m, 2H), 2.15 (s, 6H), 2.60–2.73 (m, 4H), 3.02–3.10 (m, 2H), 4.05 (t, J = 7 Hz, 2H), 5.18 (s, 2H), 6.55 (d, J = 8 Hz, 1H), 6.81 (m, 1H), 6.88–6.94 (m, 1H), 7.03–7.12 (m, 1H), 7.33–7.53 (m, 5H); MS (EI) m/z: 365.2 (M$^+$).

[4-Benzyloxy-3-(2-dimethylaminoethyl)indol-1-yl]butyric acid (**7**)

To a solution of **4** (237 mg, 0.65 mmol) in ethanol (10 mL), a solution of 10% aqueous potassium hydroxide (12 mL) was added with stirring. The mixture was heated under reflux for 10 h. The solution was acidified with 2 M hydrochloric acid and evaporated under reduced pressure. The residue was triturated with dichloromethane to dissolve the product. The solution was separated and the solvent was evaporated under vacuum giving **7** as a yellow waxy solid (97 mg, 36%). ^{1}H NMR (dimethylsulfoxide-d$_6$): 2.02 (quint, J = 7 Hz, 2H), 2.29 (t, J = 7 Hz, 2H), 2.64 (s, 6H), 3.21–3.32 (m, 4H), 4.21 (t, J = 7 Hz, 2H), 5.29 (s, 2H), 6.72–6.78 (m, 1H), 7.12–7.20 (m, 2H), 7.25 (s, 1H), 7.43–7.69 (m, 5H); MS (EI) m/z: 380.2 (M$^+$).

4-[3-(2-Dimethylaminoethyl)-4-hydroxyindol-1-yl]butyric acid (**8**)

A solution of **7** (75 mg, 0.18 mmol) in 95% ethanol (10 mL) was added to 40 mg Pd/C (10%, w/w) in a hydrogenation flask. The mixture was stirred under a balloon filled with H$_2$ for 2 h at room temperature. The catalyst was removed by filtration through a cotton pad under nitrogen. The solvent was evaporated under reduced pressure giving **8** as a waxy solid (49.7 mg, 95%). IR (KBr): 2900, 1720, 1460,

1260, 770, 730 cm^{-1}; ^{1}H NMR (CH$_3$OD): 2.05 (quint, J = 7 Hz, 2H), 2.25 (t, J = 7 Hz, 2H), 2.89 (s, 6H), 3.22–3.34 (m, 2H), 3.44–3.51 (m, 2H), 4.10 (t, J = 7 Hz, 2H), 6.41 (d, J = 7 Hz, 1H), 6.84–6.95 (m, 2H), 7.00 (s, 1H); MS (EI) m/z: 290.2 (M$^+$); HR-ESI-MS for C$_{16}$H$_{22}$N$_2$O$_3$ + H$^+$: calculated 291.1709; found 291.1738.

The HPLC system for the analysis of **8** consisted of a Hewlett-Packard Series II liquid chromatograph equipped with Hewlett-Packard Series 1050 variable wavelength detector. Separation was achieved on a Merck LiChrospher 60 RP select B column, 4 mm (internal diameter) × 250 mm, particle size 5 μm. The mobile phase consisted of 0.02 M KH$_2$PO$_4$/acetonitrile (gradient from 95:5 to 75:25, v/v). The flow rate was 1 mL min^{-1} and the injected sample volume was 20 μL. The UV absorbance was monitored at 266 nm. The purity of **8** was greater than 90%.

Preparation of the immunogen

Dry chloroform (3.6 mL) was added to polymer-supported EDAC (383 mg) under nitrogen. A solution of **8** (10.4 mg, 0.036 mmol) in dry DMF (0.9 mL) was then added, followed by the addition of a solution of *N*-hydroxysuccinimide (3.7 mg, 0.032 mmol) in dry DMF (0.9 mL). The reaction mixture was stirred for 40 h. The polymer was removed by filtration through a glass filter. The filtrate was concentrated under reduced pressure to remove the chloroform and then added to a solution of BSA (148 mg, 0.002 mmol) in phosphate buffer (0.1 M, pH 7.0, 15 mL) under nitrogen. After stirring overnight, the mixture was purified by gel chromatography using PD10 desalting columns from Amersham Pharmacia Biotech (Braunschweig, Germany). Elution was performed with 0.1 M phosphate buffer (pH 7.0). The eluate was dialysed against bidistilled water (3 days). The water was then removed by lyophilization to yield 122 mg of the hapten–BSA conjugate. The conjugate was analysed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) employing a TofSpec 2E Spectrometer from Micromass (Manchester, UK). The matrix was sinapinic acid. The protein sample was dissolved in 0.1% trifluoroacetic acid

(1.8 mg/500 μL); 0.5 μL of the sample solution was spotted with 0.5 μL of the matrix solution onto the MALDI target.

Results and Discussion

A specific antibody is needed for the development of an immunoassay. With a molecular weight of 204, psilocin is too small to initiate an immune response on its own. Antibodies for psilocin can only be raised against a protein–hapten conjugate, whereby the hapten should have high similarity with psilocin. Characteristic structural features of psilocin are the phenolic hydroxy group in position 4 and the (dimethylamino)ethyl side chain in position 3 of the indole ring system. In the immunogen, these structures should be accessible for antibody generation.

We therefore decided to prepare psilocin derivatives with an alkyl spacer in position 1 ending with a primary amine. The coupling of this hapten to the protein should be achieved via glutaraldehyde condensation.

The synthesis started from 4-benzyloxy-*N,N*-dimethyltryptamine (**1**). This was reacted with NaH and dibenzyl-(3-bromopropyl)amine in DMF to yield **2** (Figure 2). Subsequently, the benzyl-protecting groups at the phenolic hydroxy moiety and the amine functionality of this compound should be removed in one step by hydrogenolysis at atmospheric pressure with Pd/C as a catalyst. Surprisingly, a mixture of several products was obtained during this reaction as shown by TLC and HPLC analysis. In the ^{1}H NMR spectrum of this mixture, signals for the propyl spacer and the dimethylaminoethyl side chain were detectable in the expected integration ratio. However, in comparison with these signals, the integrals for the aromatic protons were not high enough. This result indicated that oxidation of the phenolic ring system had probably occurred.

Since the hydrogenolysis of the benzyl-protecting groups of the primary amine moiety of **2** lasts much longer than the deprotection of the phenolic group, we decided to generate the free ω-aminoalkyl side chain before cleaving the 4-benzyloxy moiety. This would offer the opportunity
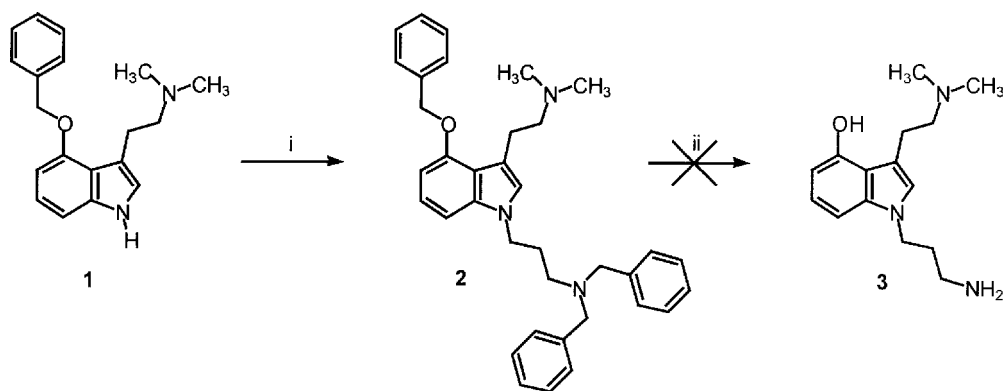

Figure 2 Synthesis of compounds **2** and **3**. i. Dibenzyl (3-bromopropyl)amine, NaH, DMF; ii. Pd/C, H$_2$, MeOH, 48 h.
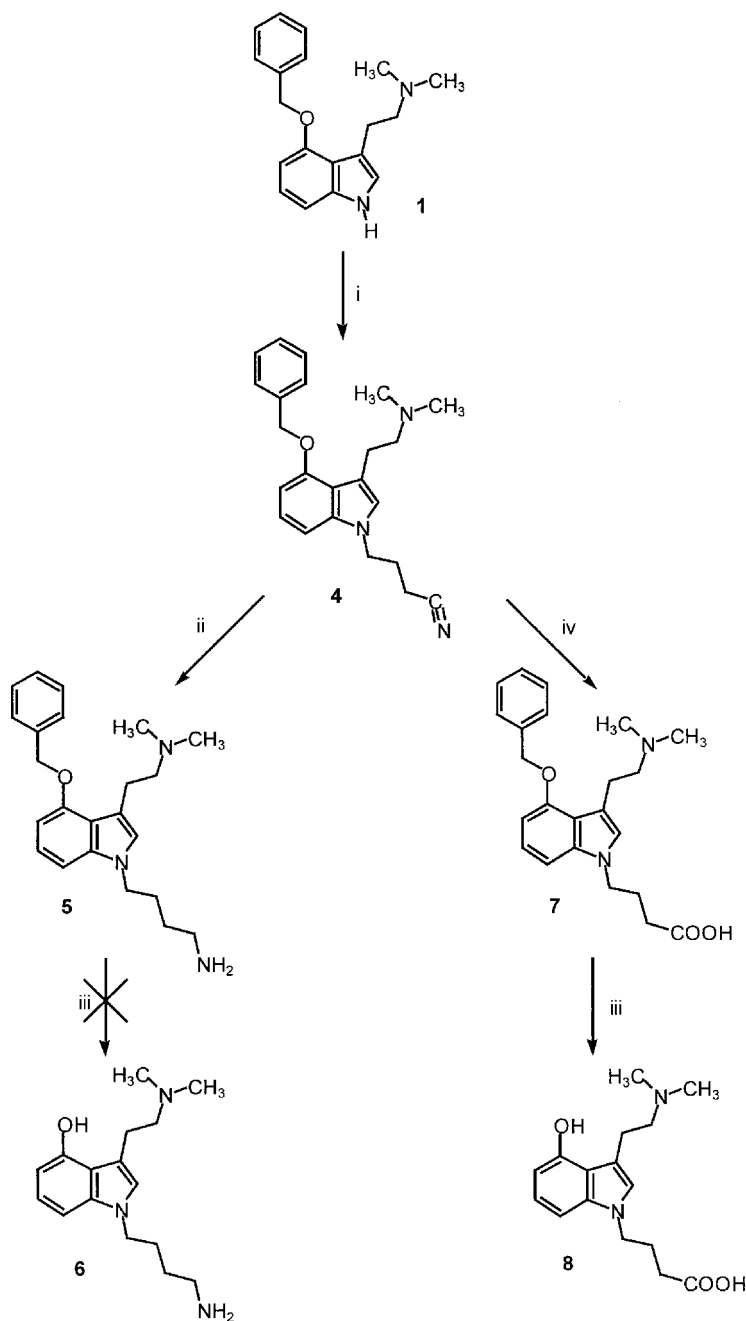
Figure 3   Synthesis of compounds **4**, **5**, **6**, **7** and **8**. i. 4-Bromobutyronitrile, NaH, DMF; ii. Raney-Nickel, H$_2$, MeOH, 38 h; iii. Pd/C, H$_2$, MeOH, 2 h; iv. aqueous KOH, ethanol.

to reduce the reaction time in the last step, probably avoiding greater decomposition of the target compound. In order to synthesize a 4-benzyloxy-*N*,*N*-dimethyltryptamine with a 1-(4-aminobutyl) side chain at the indole nitrogen, a 3-cyanopropyl residue was introduced at the indole nitrogen first by reaction of **1** with 4-bromobutyronitrile in DMF in the presence of NaH as base (Figure 3). The nitrile moiety of **4** was then converted to a primary amine (**5**) by catalytic hydrogenation with Raney-Nickel as a catalyst. The benzyl-protecting group was not cleaved

under the conditions applied. Finally, deprotection of the phenolic hydroxy group was carried out by hydrogenolysis using Pd/C as a catalyst. However, the reduction of the reaction time in this last step (2 h instead of 48 h) did not lead to a pure compound **6**. Similar side products were obtained as during the synthesis of **3**.

Finally, we decided to prepare a psilocin derivative with a 1-alkyl spacer ending with a carboxy group. The carboxylic acid moiety would offer the possibility to couple the hapten to BSA via an activated ester. Thus, the nitrile
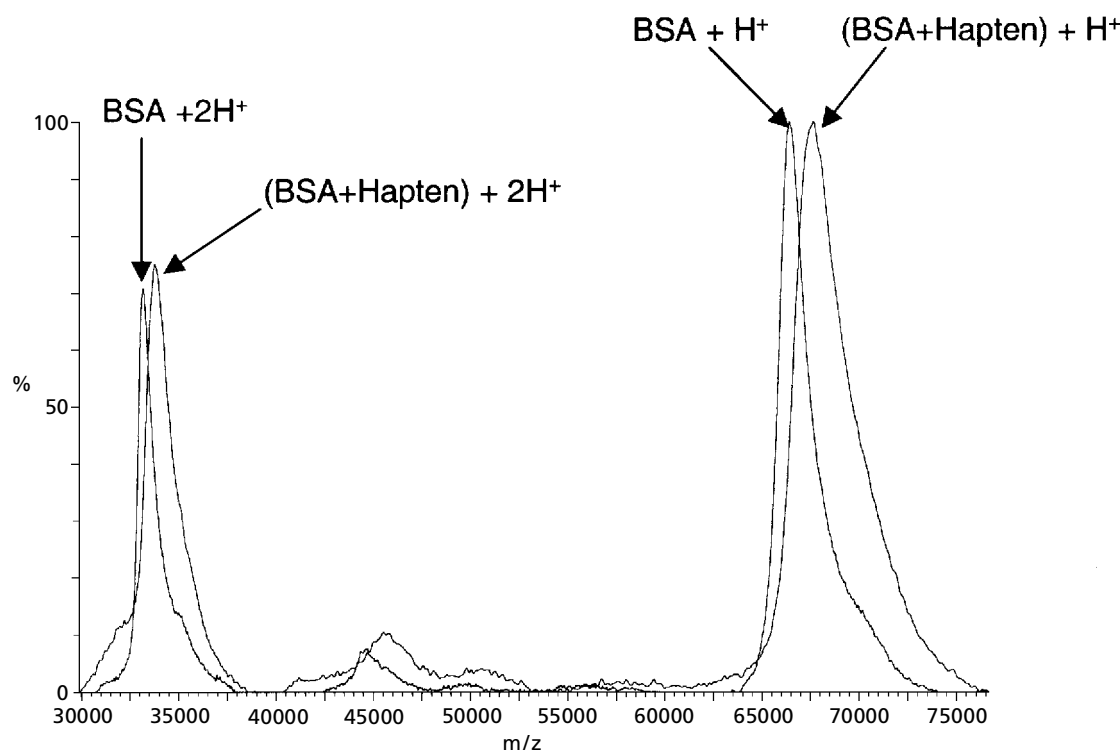
Figure 4   Matrix-assisted laser desorption ionization mass spectra of bovine serum albumin (BSA) and the BSA–hapten conjugate.

functionality of **4** was hydrolysed to a carboxy group with KOH (Figure 3). The obtained intermediate **7** was then converted to the target **8** by cleavage of the benzylether with Pd/C-hydrogen. Decomposition of this substance during the reaction did not occur, as shown by HPLC analysis.

The coupling of a hapten via a carboxylic acid group to the amino groups of a protein is usually performed directly using EDAC or N,N'-dicyclohexylcarbodiimide (Jenner & Law 1996). This process, however, leads to undesirable side reactions such as cross-linking of the protein and N-acylurea formation on the protein (Adamczyk et al 1995). These disadvantages can be avoided by the use of pure active esters. Adamczyk et al (1995) described a method to produce pure N-hydroxysuccinimide and pentafluorophenol esters from carboxylic acid haptens by the application of polymer-supported EDAC. We used this method to generate the N-hydroxysuccinimide ester of the psilocin derivative **8**. After activation with solid-supported EDAC, the EDAC-activated ester of **8** was reacted with N-hydroxysuccinimide. The polymer-bound EDAC was removed by filtration through a glass filter and the filtrate containing the active N-hydroxysuccinimide ester of **8** was used without further purification. For coupling to BSA, the solution of the activated hapten was added to a solution of BSA in phosphate buffer in a molar ratio of 18:1. This mixture was stirred overnight and the BSA–hapten conjugate was purified by gel chromatography on a Sephadex column, followed by dialysis. The solvent was removed by lyophilization.

Since the maximum of the UV absorbance of psilocin and the generated hapten is not distinct from that of the protein, UV spectroscopic determination of the ratio of incorporation of the hapten into BSA was not possible. Therefore, we used MALDI-MS for the characterization of the bioconjugate (Adamczyk et al 1994). Mass spectra were successfully recorded for BSA and the BSA–hapten conjugate (Figure 4). For BSA and the conjugate, molecular weights of 66 497 and 67 682 Da were measured, respectively. This indicates an average incorporation ratio of 4–5 molecules of psilocin hapten (MW 290) per molecule of BSA.

In conclusion, a psilocin–BSA immunogen was prepared, which can be used to develop a radioimmunoassay for psilocin in human plasma.

References

Adamczyk, M., Buko, A., Chen, Y. Y., Fishpaugh, J. R., Gebler, J. C., Johnson, D. D. (1994) Characterization of protein-hapten conjugates. 1. Matrix-assisted laser desorption ionization mass spectrometry of immuno BSA-hapten conjugates and comparison with other characterization methods. *Bioconjug. Chem.* **5**: 631–635

Adamczyk, M., Fishpaugh, J. R., Mattingly, P. G. (1995) An easy preparation of hapten active esters via solid supported EDAC. *Tetrahedron Lett.* **36**: 8345–8346

Beug, M. W., Bigwood, J. (1981) Quantitative analysis of psilocybin and psilocin in *Psilocybe baeocystis* by high-performance liquid chromatography and by thin-layer chromatography. *J. Chromatogr.* **207**: 379–385

Bogusz, M. J. (2000) Liquid chromatography-mass spectrometry as a routine method in forensic sciences: a proof of maturity. *J. Chromatogr. B* **748**: 3–19

Carter, N., Rutty, G. N., Milroy, C. M., Forrest, A. R. W. (2000) Deaths associated with MBDB misuse. *Int. J. Legal Med.* **113**: 168–170

Christiansen, A. L., Rasmussen, K. E., Tønnessen, F. (1981) Determination of psilocybin in *Psilocybe semilanceata* using high-performance liquid chromatography on a silica column. *J. Chromatogr.* **210**: 163–167

De Letter, E. A., Coopman, V. A. E., Cordonnier, J. A. C. M., Piette, M. H. A. (2001) One fatal and seven non-fatal cases of 4-methyl-thioamphetamine (4-MTA) intoxication: clinico-pathological findings. *Int. J. Legal Med.* **114**: 352–356

Gartz, J. (1986) Detection of tryptamine derivatives in fungi of the genera *Gerronema*, *Hygrocybe*, *Psathyrella* and *Inocybe*. *Biochem. Physiol. Pflanz.* **181**: 275–278

Grieshaber, A. F., Moore, K. A., Levine, B. (2001) The detection of psilocin in human urine. *J. Forensic Sci.* **46**: 627–630

Hasler, F., Bourquin, D., Brenneisen, R., Bär, T., Vollenweider, F. X. (1997) Determination of psilocin and 4-hydroxyindole-3-aceticacid in plasma by HPLC-ECD and pharmacokinetic profiles of oral and intravenous psilocybin in man. *Pharm. Acta Helv.* **72**: 175–184

Hofmann, A., Troxler, F. (1959) Identification of psilocin. *Experientia* **15**: 101–102

Hofmann, A., Frey, A., Ott, H., Petrzilka, T., Troxler, F. (1958) The structure and synthesis of psilocybin. *Experientia* **14**: 397

Horita, A., Weber, L. J. (1961) The enzymic dephosphorylation and oxidation of psilocybin and psilocin by mammalian tissue homogenisates. *Biochem. Pharmacol.* **7**: 47–54

Horita, A., Weber, L. J. (1962) Dephosphorylation of psilocybin in the intact mouse. *Toxicol. Appl. Pharmacol.* **4**: 730–737

Jenner, W. N., Law, B. (1996) Immunogen preparation and purification. In: Law, B. (ed.) *Immunoassay – a practical guide.* London, Taylor & Francis Ltd, pp 11–31

Kalberer, F., Kreis, W., Rutschmann, J. (1962) The fate of psilocin in the rat. *Biochem. Pharmacol.* **11**: 261–269

Keller, T., Schneider, A., Regenscheidt, P., Dirnhofer, R., Rücker, T., Jaspers, J., Kisser, W. (1999) Analysis of psilocybin and psilocin in *Psilocybe subcubensis* Guzmán by ion mobility spectrometry and gas chromatography-mass spectrometry. *Forensic Sci. Int.* **99**: 93–105

Kysilka, R. (1990) Determination of psilocin in rat urine by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **534**: 287–290

Lindenblatt, H., Krämer, E., Holzmann-Erens, P. Gouzoulis-Mayfrank, E., Kovar, K. A. (1998) Quantitation of psilocin in human plasma by high-performance liquid chromatography and electrochemical detection: comparison of liquid-liquid extraction with automated on-line solid-phase extraction. *J. Chromatogr. B* **709**: 255–263

Musshoff, F., Madea, B., Beike, J. (2000) Hallucinogenic mushrooms on the German market – simple instructions for examination and identification. *Forensic Sci. Int.* **113**: 389–395

Nagle, A. S., Salvatore, R. N., Chong, B. D., Jung, K. W. (2000) Efficient synthesis of β-amino bromides. *Tetrahedron Lett.* **41**: 3011–3014

Nichols, D. E., Frescas, S. (1999) Improvements to the synthesis of psilocybin and a facile method for preparing the *O*-acetyl prodrug of psilocin. *Synthesis* **6**: 935–938

Pedersen-Bjergaard, S., Sannes, E., Rasmussen, K. E., Tønnesen, F. (1997) Determination of psilocybin in *Psilocybe semilanceata* by capillary zone electrophoresis. *J. Chromatogr. B* **694**: 375–381

Semerdzieva, M., Wurst, M., Koza, T., Gartz, J. (1986) Psilocybin in fruiting-bodies of *Inocybe aeruginascens*. *Planta Med.* **52**: 83–85

Sottolano, S. M., Lurie, I. S. (1983) The quantitation of psilocybin in hallucinogenic mushrooms using high performance liquid chromatography. *J. Forensic Sci.* **28**: 929–935

Sticht, G., Käferstein, H. (2000) Detection of psilocin in body fluids. *Forensic Sci. Int.* **113**: 403–407

Stijve, T., Kuyper, T. W. (1985) Occurrence of psilocybin in various higher fungi from several European countries. *Planta Med.* **51**: 385–387

Wurst, M., Kysilika, R., Koza, T. (1992) Analysis and isolation of indole alkaloids of fungi by high-performance liquid chromatography. *J. Chromatogr.* **593**: 201–208

Yamada, F., Tamura, M., Somei, M. (1998) A facile synthesis of psilocin from indole-3-carbaldehyde. *Heterocycles* **49**: 451–457