Pyrrolizidine alkaloids from *Symphytum officinale* L. and their percutaneous absorption in rats

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Summary. An analysis of a commercial sample of Symphyti radix originating from Poland with a total alkaloid content of 0.07% revealed the presence of 7 pyrrolizidine alkaloid-N-oxides: 7-acetyl intermedine, 7-acetyl lycopsamine as the main constituents and lycopsamine, intermedine, symphytine and traces of 2 further not yet identified alkaloids. The percutaneous absorption of these alkaloids was investigated in rats, using a crude alcoholic extract of the plant corresponding to a dose of 194 mg alkaloid-N-oxides/kg b.wt. The excretion of N-oxides in the urine during 2 days was in the range of 0.1–0.4% of the dose. The dermally absorbed N-oxides are not or only to a small extent converted to the free alkaloids in the organism. The oral application led to a 20–50 times higher excretion of N-oxides and free alkaloids in the urine.

Symphytum officinale L. (comfrey) is a member of the plant family of Boraginaceae and is widely used by humans as a medicinal herb. It has been shown by Furuya et al.[2,3] to contain the pyrrolizidine alkaloids, (PA), symphytine and echimidine. A long term carcinogenicity study performed by Hirono et al.[4] proved that roots and leaves of *Symphytum officinale* L. added to the diet of rats can induce hepatocellular adenomas. The doses fed were 8, 4, and 2% of the diet for the roots and 33, 16, and 8% for the leaves. Hepatocellular adenomas have also been induced by feeding the pure pyrrolizidine alkaloid symphytine[5]. A recent study by Culvenor et al.[6] on the effects of comfrey on rats assaying for total plasma proteins, GOT and GLDH showed impairment of the liver function.

The medicinal application of comfrey is mainly external, as an ointment to cure muscle pains and inflammations of the veins. The present paper gives further data about the chemical identification of pyrrolizidine alkaloids in *Symphytum officinale* L. and their percutaneous absorption in rats.

Experimental. Isolation of the alkaloids. 1.7 kg of ground roots of *Symphytum officinale* L. (Radix consolidae, Symphyti radix, supplied by Siegfried, Zofingen, Switzerland, origin Poland) was extensively extracted with methanol in a Soxhlet apparatus. The solvent was partly removed under reduced pressure, the white precipitate (allantoin) filtered off and discarded. 1 N hydrochloric acid was added to the residue and the aqueous acid solution reduced by stirring with zinc dust overnight. The alkaloids were extracted with chloroform from the alkalized aqueous solution. Evaporation of the solvent yielded a crude red-brown oil (1.7 g) which did not crystallize. The alkaloids were separated and further purified using preparative TLC (precoated silicagel plates 60F254, 0.25 mm, supplied by Merck, Darmstadt) and $CHCl_3$–CH_3OH–NH_3 (25%) 60:10:1 as eluting agent. 3 zones (R_f-values: 0.48, 0.43 (zone B) and 0.12) giving a positive Mattocks-reaction[7] were scratched off and eluted with methanol. The oily residues were analyzed by GC-MS or hydrolyzed with barium hydroxide.

Hydrolysis of pyrrolizidine alkaloids. The purified pyrrolizidine alkaloids of zone B (104 mg) were added to an aqueous solution of 200 mg barium hydroxide and refluxed for 1 h. After cooling, the solution was saturated with CO_2 and the precipitated barium carbonate removed by filtration. The solution was acidified to congo red and continuously extracted with ether for 24 h. The acidic residue of the organic layer was further purified by preparative TLC with butanol-ammonia-water (30:1:5) and recrystallized from benzene/light petroleum. The aqueous layer remaining after the extraction of the acids was made alkaline with an excess of ammonia (25%) to pH 9.5, and was extracted on an Extrelut column using the standard procedure[8] eluting agent chloroform-methanol 9:1. Evaporation of the solvent and recrystallization from acetone yielded colorless needles.

GC-MS-conditions. The separation and identification of the alkaloids was carried out on a Finnigan Model 4021 GC-MS system. GC was performed on a 20 m SE54 capillary column, with He as the carrier gas. The tempera-

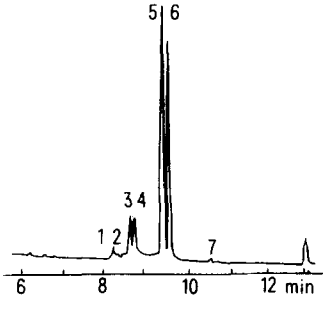

Figure 1. The PA of Symphyti radix: 1, 2 not identified PAs; 3 intermedine, 4 lycopsamine mol. wt of 299; 5 7-acetylintermedine mol. wt 341; 6 7-acetyllycopsamine mol. wt 341; 7 symphytine mol. wt 381.

Table 1. Excretion of PA and derivatives in the urine of rats, during 48 h, treated with 194 mg/kg b.wt *Symphytum* PA-N-oxides

Identified compound	% of applied dose			
	Oral		Dermal	
	Rat 1	Rat 2	Rat 3	Rat 4
7-Ac-intermedine/7-Ac-lycopsamine	3.7	1.2	n.d.	n.d.
7-Ac-intermedine/7-Ac-lycopsamine N-oxides	0.2	0.2	0.4	0.1
Intermedine/lycopsamine	4.1	1.4	n.d.	n.d.
Intermedine/lycopsamine N-oxides	1.4	0.1	n.d.	n.d.

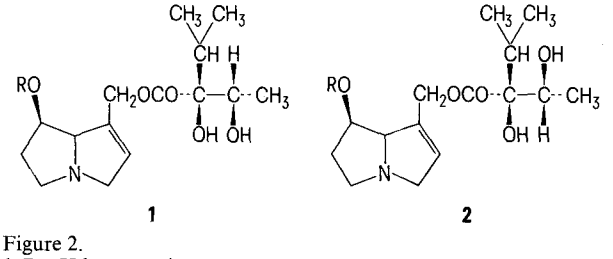

Figure 2.
1 R = H lycopsamine
1 R = CH_3CO–, 7-acetyllycopsamine
1 R = $CH_3CH{=}CCH_3{-}CO$–, symphytine
2 R = H, intermedine
2 R = CH_3CO–, 7-acetylintermedine

Table 2. Summary of PA found in *Symphytum officinale* L. and *Symphytum* × *uplandicum* Nyman

	Culvenor et al.[14,15]	Furuya et al.[2,3]	Huizing et al.[16]	Tittel et al.[12] and Wagner et al.[13]	Alkaloids as identified in this paper
Symphytine	+	+ +	+	+ +	+
Echimidine	+ +	+ +	−	+ +	−
7-Acetyllycopsamine	+ +	−	+ +	+ +	+ +
7-Acetylintermedine	+	−	+ +	−	+ +
Lycopsamine	+ +	−	+ +	+ +	+
Intermedine	+	−	+	−	+
Symlandine	+	−	−	−	−
Uplandicine (resp. N-oxides)	+ +	−	−	−	−

+ + Main components; + minor components.

tures were injector 250 °C and column 100 °C for 1 min and 10 °C/min until 220 °C. The mass spectrometer had an electron energy of 70 eV and ion source temperature of 250 °C. CI conditions with methane and ammonia as reactant gas were: source pressure 0.30 torr, and source temperature 200 °C. Quantification of the alkaloids was made by peak integration.

Oral and dermal administration of Symphytum alkaloids to rats. 1st experiment. 4 Sprague Dawley derived/SIV 50 male rats (200 g each) were kept individually in polyethylene metabolism cages with free access to a commercial diet (NAFAG, Switzerland) and water. Urine was collected at 24-h intervals during 4 days. 52 mg of reduced crude *Symphytum* alkaloids were dissolved in 1 ml 0.05 M acetic acid. 2 rats were used; each received 0.5 ml of this solution by gavage. For the dermal application 0.25 ml of an ethanolic solution containing 26 mg of reduced crude *Symphytum* alkaloids were administered on the backs of 2 animals. The area destined for treatment measured about 3 cm^2 and was shaved with an electric clipper immediately prior to application. After treatment it was covered with an aluminium foil and fixed with an elastic bandage. The contact time was 44 h. The urine of the treated animals was collected daily. The urine samples were made alkaline by addition of aqueous ammonia, and were extracted on an extrelut column Extrelut® (Merck) using the standard procedure with 40 ml chloroform-methanol 9:1. The alkaloids in the organic layer were identified with TLC and Mattocks[7] procedure to make the spots visible. The aqueous layer was recovered from the column and was incubated with glucuronidase/arylsulfatase (Boehringer, Germany) overnight, and subsequently reduced with zinc/hydrochloric acid. The obtained alkaloids were purified and analyzed as described before.

2nd experiment. Application of the *Symphytum* alkaloid N-oxides. A dose corresponding to 31 mg/rat (194 mg alkaloid-N-oxides/kg b.wt) of the crude unreduced *Symphytum* extract was given orally to 2 rats and the same dose was applied dermally after shaving to 2 further animals. Collection and analysis of the urine samples were performed as described in the 1st experiment, but quantitation of the alkaloids found in the urine was done with GC-FID and the identification with GC-MS.

Results. a) Identification of pyrrolizidine alkaloids in *Symphytum officinale* L. The *Symphytum* alkaloids isolated from a sample of Polish origin were separated by glass capillary GC and characterized by mass spectrometry. 7 peaks were found (fig. 1) giving an EI-MS fragmentation with m/z = 93–95, 119–121, 136 and 138; typical for pyrrolizidine alkaloids containing an aminoalcohol of the type retronecine in the molecule. The 2 main components (0.056% of dry drug) had almost the same retention time (peaks Nos 5 and 6 in fig. 1), both with a mol. wt of 341 and identical MS-fragmentation: the base peak m/z = 180 corresponding to $C_{10}H_{14}NO_2$ and m/z = 281 (M-60, CH_3CO_2H) gives strong evidence for the presence of an acetyl group at 0-7 (Pedersen[9]). Cochromatography with the authentic samples of 7-acetyl-intermedine and 7-acetyl-lycopsamine (kindly provided by Dr C.C.J. Culvenor) and comparison of the mass spectra finally proved the presence of these 2 alkaloids (fig. 1) Hydrolysis of a mixture of these 2 compounds (zone B on TLC) yielded retronecine, m.p. 117–118 °C, colorless needles undepressed in addition to authentic compound and 2 nonvolatile acids. One of them was isolated and had a m.p. of 120–124 °C after recrystallization from benzene/light petroleum. ^1H NMR ($CDCl_3$/CD_3OD, 90 MHz): 0.92 (3H, d, J = 6.5 Hz), 0.95 (3H, d, J = 6.5 Hz), 1.25 (3H, d, J = 6.5 Hz), 2.1 (1H, m), 4.0 (1H, q, J = 6.5). Viridifloric acid: m.p. 119–127 °C (10), ^1H NMR: 0.92 (3H, d, J = 6.8 Hz), 0.95 (3H, d, J = 6.6 Hz), 1.23 (3H, d, J = 6.8), 2.1 (1H, m), 4.07 (1H, q, J = 6.8)[10].

Peaks Nos. 3 and 4 (fig. 1) showed identical mass spectra of the 2 diastereomers 5 and 6. This makes an identification as lycopsamine (0.007% of dry weight drug)[10] and intermedine (0.007% of dry wt drug)[11] almost certain. Only traces of symphytine with a $M^+ = 381$ were found in the sample (peak No. 7, fig. 1). The EI- and CI-mass spectra of peaks Nos 1 and 2 indicate the presence of 2 further not yet identified pyrrolizidine alkaloids. Echimidine was not found in our sample[2,3].

b) Percutaneous and gastrointestinal absorption of *Symphytum* alkaloids. Rats treated with 130 mg/kg b.wt. of reduced *Symphytum* alkaloids by gavage as well as the dermally treated rats excreted the compounds 7-acetyllycopsamine, 7-acetyl-intermedine, lycopsamine, intermedine and retronecine in the 1st day urine. An estimation by TLC showed a) that a deacetylation of 7-acetyl-lycopsamine and 7-acetyl-intermedine takes place in the organism and b) that the dermally treated rats excreted about 20 times less alkaloids in the urine than the rats dosed by gavage.

c) Percutaneous and gastrointestinal absorption of the Symphytum alkaloid N-oxides. Table 1 shows the excretion pattern of alkaloids in the urine after application of the same dose of *Symphytum* alkaloid N-oxides by different routes of administration.

Discussion. Earlier analyses of *Symphytum officinale* and hybrids[12–16] are summarized in table 2. Quantitatively the most important pyrrolizidine alkaloids found are 7-acetyllycopsamine, 7-acetylintermedine, echimidine and symphytine. Although there is not much doubt that all these compounds possess genotoxic activity, only symphytine has so far been tested in a long-term study for carcinogenicity, and even these data are not sufficient to draw conclusions about the carcinogenic potency. To perform a risk assessment further data about the mutagenicity and carcinogenicity of the compounds mentioned above are needed. The present study shows that the 2 diastereomers 7-acetyllycopsamine and 7-acetylintermedine as well as their N-oxides

can be absorbed percutaneously although to a considerable smaller extent than by ingestion. The individual differences from rat to rat are considerable. The following conclusions can be drawn from the experiment: a) the 2 N-oxides of the isomers 7-acetyl-lycopsamine and 7-acetyl-intermedine are metabolized to a great extent to the free alkaloids and to the deacetylated forms in orally-treated animals, b) no reduction of N-oxides took place by dermal application of the compounds within the limits of detection, c) the percutaneous absorption of N-oxides is smaller by a factor of 20–50 compared to the gastrointestinal absorption when the excretion of N-oxides and metabolites in the urine is taken as a measure.

In our samples the *Symphytum* alkaloids were present almost completely as N-oxides. The dermally absorbed PA-N-oxides are not or only to a small extent converted to the free alkaloids in the organism. This conversion seems to be an essential step for the toxic action of PA-N-oxides[17,18]. Our data are in agreement with the findings of Powis et al.[19] that the gut flora plays a major role in the metabolic reduction of PA-N-oxides. This difference in the metabolism together with the small degree of dermal absorption makes it likely that the occasional external use of *Symphytum* preparations should not be hazardous.

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Conjugates of adenine 9-α-D-arabinofuranoside monophosphate (ara-AMP) with lactosaminated homologous albumin are not immunogenic in the mouse[1]

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Summary. Conjugates of adenine-9-β-D-arabinofuranoside (ara-A) or of ara-A monophosphate (ara-AMP) with asialofetuin or with heterologous lactosaminated serum albumin (L-SA) are strong antibody inducers. But ara-AMP conjugates prepared with homologous L-SA are not immunogenic, at least in mice.

In the therapy of some diseases (e.g. tumors, infections caused by intracellular microorganisms) drug side effects might be circumvented by coupling the drug to a protein carrier which is selectively taken up by the cells where the pharmacological action is required. If the bond between the drug and the vector is broken down in lysosomes, the drug should be released free and concentrated in the cells into which it was transported[2–5]. With the aim of reducing side effects occurring in the treatment of chronic hepatitis B with adenine-9-β-D-arabinofuranoside (ara-A)[6–8], this drug and its monophosphate derivative (ara-AMP) were coupled to asialofetuin (AF)[9] and to lactosaminated serum albumin (L-SA)[10]. These galactosyl-terminating glycoproteins are internalized only in hepatocytes where they are delivered to lysosomes[11–14].

After injection of AF or L-SA conjugates in mice with hepatitis caused by *Ectromelia* virus, ara-A and ara-AMP are selectively concentrated, in a pharmacologically active form, into hepatocytes[9,10]. L-SA-ara-AMP conjugates do not display acute toxic effects at least in mice (unpublished experiments). A conjugate L_{31}(human)SA-ara-AMP_{10}, administered at a dose of 10 μg per 1 g b.wt, produced a 50% inhibition of virus DNA synthesis in liver of *Ectromelia* virus infected mice; the same conjugate, injected at a dose 11 times higher (the maximum tested), did not cause any sign of toxicity in mice. However, these conjugates might be immunogenic and consequently produce allergic lesions. By using homologous (i.e. of same species) L-SA this risk can be reduced[9,10] but not excluded a priori since antibodies are produced against some hapten-homologous albumin conjugates[15].

In the present experiments we studied whether ara-AMP and ara-A conjugates prepared with AF or with homologous or heterologous L-SA cause a humoral response and/or a delayed-type hypersensitivity in mice.

Materials and methods. Fetuin (Sigma type III) was desialylated by neuraminidase[16]. Lactose was coupled to ε-NH_2 of lysine residues of human (HSA) (crystalline), rabbit (RSA) (crystalline) and mouse serum albumin (MSA) (fraction V) by reductive amination with cyanoborhydride[17,18]. All SA were from the Sigma Chemical Company. In different preparations of L-SA, increasing amounts of lactose were coupled as a function of time of reaction[13–18]. Ara-AMP (Warner-Lambert) was conjugated to AF and to L-SA by the use of 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide (Fluka)[9,10]. In this coupling, conjugation probably takes place mostly be the formation of an amide bond between the ε-NH_2 group of lysine in the protein and the phosphate