distorted octahedral environment with idealized S_2 symmetry, is bound to six nitrogen (N1) atoms. The As–N1 distances span 1.920(3) – 1.938(2) Å and the N1-As-N4 angles lie between 88.2(1) – 91.8(1)°. While the N1–N2 bond lengths range 1.222(3) – 1.238(3) Å, the terminal N2–N3 lengths range 1.120(3) – 1.123(3) Å and are both in good accord with the bond lengths of bis(trifluoromethyl)arsinic azide[2a] and trifluoromethylarsinic diazide.[2b] The N1-N2-N3 angle lies within 173.6(3) – 176.3(3)° and in good agreement with N-N-N angles of other previously reported covalent azides.[10] The angle between the arsenic atom and the azide group (As-N1-N2) is 116.2(2)°.

Compound **1** shows only moderate impact, friction, and electrostatic sensitivity but explodes when subject to a thermal shock test.[11] The relatively high kinetic stability of **1** can be explained by the fact that the large and bulky $[PPh_4]^+$ ions separate the $[As(N_3)_6]^-$ ions in the solid state and therefore cause a high activation barrier for the overall exothermic decomposition. This phenomenon has also been observed for the $[I(N_3)_2]^-$ ion, which is kinetically stable as the $[PPh_4]^+$ salt but highly explosive as the $[NMe_4]^+$ salt.[12]

Experimental Section

Caution: Covalent and ionic azides as well as arsenic compounds are very toxic and covalent azides are potentially explosive. Appropriate safety precautions should be taken.

Apparatus: NMR: Jeol EX400 Delta (^{1}H, ^{13}C: relative to $\delta_{TMS} = 0.00$; ^{31}P: relative to $\delta_{H_3PO_4} = 0.00$; ^{14}N: relative to $\delta_{CH_3NO_2} = 0.00$; ^{75}As: relative to $\delta_{KAsF_6} = 0.00$). IR: Nicolet 520 FT IR. Raman: Perkin Elmer Spectrum 2000R NIR FT. Elemental (C, H, N) analysis: Analysator Elementar Vario EL.

Compound **1** was synthesized under an inert gas atmosphere at 25 °C by the slow addition of neat $(CH_3)_3SiN_3$ (0.53 mL, 4 mmol) with stirring to a solution of $[PPh_4][AsCl_6]$ (0.314 g, 0.5 mmol) in CH_2Cl_2 (25 mL). After 5 hours the solvent and $(CH_3)_3SiCl$ were removed by a dynamic vacuum. Colorless crystals were obtained after recrystallization from CH_2Cl_2 at –25 °C. Yield: 0.237 g (71 %, based on $[PPh_4][AsCl_6]$); elemental analysis for $C_{24}H_{20}AsN_{18}P$: calcd: C 43.25, H 3.02, N 37.83; found: C 42.89, H 2.91, N 37.44; IR (powder between CsI plates): $\tilde{\nu}$: 2085 (vs, $\nu_{as}(N_3)$), 1585 (w), 1481 (m), 1434 (s), 1270 (s, $\nu_s(N_3)$), 1108 (s), 996 (w), 888 (m), 783 (s), 688 (s, $\delta(N_3)$), 526 (s), 416 (s, ν(AsN)), 305 cm^{-1} (s); Raman (525 scans, 200 mW, 180° geometry, 25 °C): $\Delta\tilde{\nu}$: 3068 (6.5), 2110 ($\nu_{as}(N_3)$ in plane, 3.5), 2084 ($\nu_{as}(N_3)$ out of plane, 1.5), 1587 (4.5), 1279 ($\nu_s(N_3)$, 3.5), 1187 (0.5), 1163 (0.5), 1099 (1.5), 1027 (2.5), 1001 (6.5), 681 ($\delta(N_3)$, 1.5), 669 ($\delta(N_3)$, 1.5), 416 (ν(AsN), 10), 267 (1.5), 252 (1.5), 116 (8) cm^{-1}; ^{13}C NMR (101 MHz, $CDCl_3$, 25 °C): $\delta = 117.5$ (d, C1), 130.8 (d, C2), 134.4 (d, C3), 135.9 (d, C4); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 6.8 – 7.5$ (Ph); ^{31}P NMR (109 MHz, $CDCl_3$, 25 °C): $\delta = 23.9$ (s); ^{14}N NMR (29 MHz, $CDCl_3$, 25 °C): δ = -141.1 (N2), -165.4 (N3), -256.2 (N1); ^{75}As NMR (46 MHz, $CDCl_3$, 25 °C): $\delta = 4$.

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Preparation of Bioconjugates through an Ugi Reaction**

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Dedicated to Professor Franz Effenberger on the occasion of his 70th birthday

The specific preparation of bioconjugates, which is, in general, the covalent linking of a low molecular weight compound to a protein or an oligonucleotide or bond formation between two proteins, can be regarded as one of the most important methods in modern biochemistry.[1] Conjugates which are constructed out of a protein or oligonucleotide (for example, an enzyme or a DNA fragment) and a pigment or a radioactive compound are widely used for

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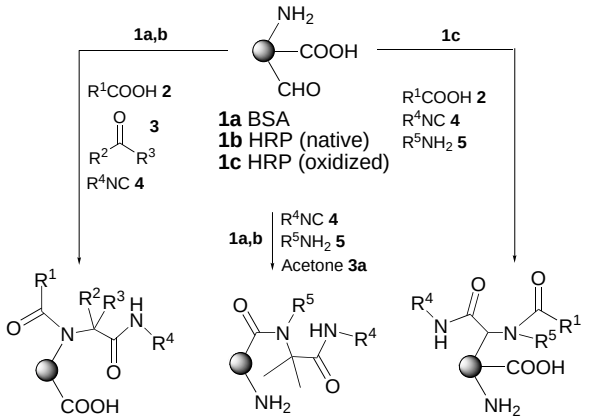
analytical and histological purposes or as devices for genetics. Antibody–enzyme conjugates are important and indispensible for almost all immunological tests. Similarly, bioconjugates, like neoglycoconjugates, which are prepared from a saccharide and a protein, or conjugates constructed from a pharmaceutically active substance, are applied as vaccines and therapeutic tools in medicine. The potential applications of bioconjugates in all these fields are legion. Although the repertory of established procedures for the preparation of bioconjugates is quite remarkable, it is still desirable to develop new methods. An overview of current procedures for the preparation of bioconjugates can be found in ref. [2].

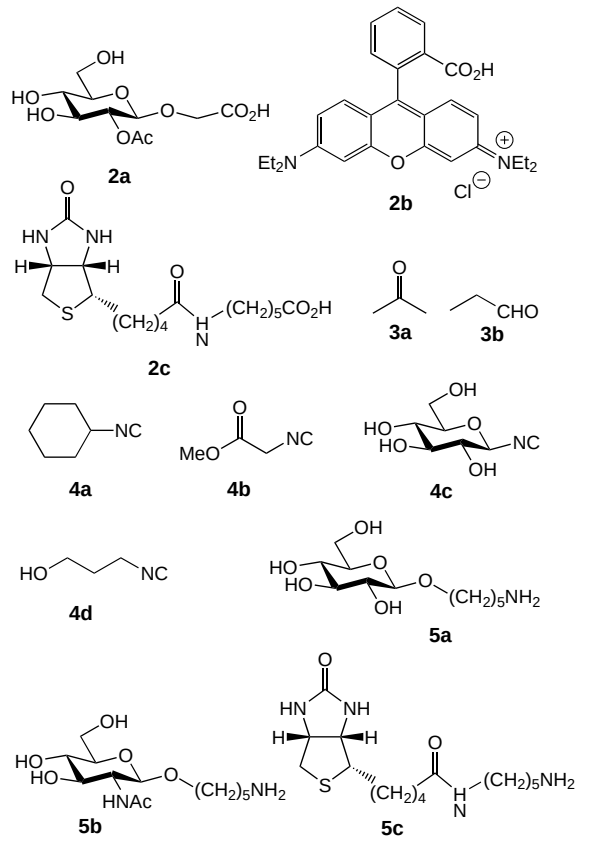
Usually, the preparation of bioconjugates from proteins is based on condensation or addition of free amino, carboxylic, thio, or aldehyde functional groups of the protein to low molecular weight compounds that also contain reactive groups. Different condensation reagents or bifunctional coupling reagents can be used for this purpose and the established standards are quite high. However, the possibilities to couple a biopolymer to several low molecular weight compounds simultaneously and in a well defined ratio is restricted. Multiple low molecular weight compounds may be conjugated to a protein by standard procedures but the exact stoichiometric ratio cannot be controlled. On the other hand, it would be highly desirable to have procedures which allow for the conjugation of multiple reporter groups to a protein in a well defined ratio.[3] Our recent work on Ugi reactions of isocyanoglycoses[4, 5] prompted us to investigate if such a multiple compound reaction can be used for the preparation of bioconjugates. In general, the Ugi reaction combines an isonitrile, an amine, a carbonyl compound, and a carboxylic acid in a fixed ratio to afford a peptide.[6, 7] To the best of our knowledge, Ugi reactions have so far been solely applied to the immobilisation of proteins by condensation of a protein with an isonitrile-modified solid support.[8]

In order to apply the Ugi reaction to the preparation of bioconjugates of the aforementioned type we reacted two proteins, bovine serum albumin (BSA) **1a** and horseradish peroxidase (HRP) **1b**,**c**, under various conditions with different carboxylic acids **2**, carbonyl compounds **3**, isonitriles **4**, and amines **5**.[9] The free functional groups of both BSA and HRP were used in Ugi reactions, either as amine groups or carboxylic acids (for native proteins **1a**,**b**) or as aldehyde groups (for $NaIO_4$ oxidized HRP **1c** [10]), to afford bioconjugates as shown in Scheme 1.

As the representative components for these Ugi reactions we chose the compounds shown in Scheme 2. In particular, glycoside **2a**, rhodamine B **2b**, and the biotin derivative **2c** were chosen as the carboxylic acids.[11] The latter was chosen in order to demonstrate the preparation of the important class of biotinylated proteins[12] by this method. As carbonyl compounds, we used acetone **3a** and propionic aldehyde **3b** although other carbonyl compounds can also be applied as long as no denaturation of the protein occurs. As isonitrile components **4**[13] for the Ugi reactions, we chose **4a** and **4b** for heterogeneous couplings and the water soluble isonitriles **4c** and **4d** for homogeneous ones. Finally, several amines **5**[14] were used in order to demonstrate the flexibility of this method.



Scheme 1. Principle for the formation of bioconjugates from proteins **1** with low molecular weight compounds **2**–**5** through Ugi reactions.



Scheme 2. The low molecular weight compounds **2**–**5** which were used.

Table 1 shows the dependence of conjugate formation by Ugi reactions of BSA **1a** with the components **3a**, **4a**, and **5a** on the buffer, the buffer's pH value, and the concentration of the components. The obtained epitope density of the prepared conjugates (molar ratio of low molecular weight compounds and the respective protein) was determined by matrix-assisted laser desorption/ionization time of flight mass spectometry (MALDI-TOF MS).[15] Optimal conditions for high epitope densities in the bioconjugates turned out to be 0.01–0.1M phosphate buffer, pH 7.5, with an expected proportionality between epitope density and the initial ratio of **1a** and **5a**. In tris/HCl buffer, as well as at lower pH values, the obtained

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Table 1. Dependence of conjugate formation between **1a** and **3a**–**5a** on buffer, pH value, and concentration of the components.

buffer	pH value	mol. ratio **5a**/**1a**	epitope density
0.01M phosphate buffer	6.0	100/1	1.9
0.01M phosphate buffer	7.5	100/1	4.0
0.1M phosphate buffer	7.5	100/1	1.2
0.1M phosphate buffer	7.5	1000/1	6.2
0.1M phosphate buffer	7.5	2000/1	6.5
0.1M phosphate buffer	7.5	3000/1	7.7
0.1M tris/HCl buffer	7.5	100/1	1.0
0.1M tris/HCl buffer	7.5	1000/1	1.6

epitope densities were significantly lower because of probable unselective reactions of the components with the buffer or because of simple hydrolysis of the isonitrile.

Table 2 summarizes the results for the conjugations of the proteins **1** with the components from Scheme 2. Once again, the epitope density was measured by MALDI-TOF MS. Furthermore, for conjugates with rhodamine B **2b**, the epitope density was also determined photometrically by measuring the extinction of an aqueous solution of the conjugates at 575 nm. Similarly, the epitope densities of conjugates with biotin derivatives **2c** and **5c** were determined by a 2-[(4-hydroxyphenyl)azo]benzoic acid (HABA)–avidine test[16] and by a streptavidine enzyme-linked immunosorbent assay (ELISA).[17] In some cases, a photometric determination of the yield of conjugate was performed and, in the case of HRP, determination of the remaining enzymatic activity was also measured by a 2,2′-azino(3-ethyl-benzythiazoline-6-sulfonic acid) (ABTS) test.[17]

Table 2. Preparation of bioconjugates from proteins **1** by an Ugi reaction with components **2**–**5** in 0.1M phosphate buffer, pH 7.5 at 25 °C.

Entry	protein **1**	mol. ratio **2**–**5**/**1**	components **2**–**5**	*t* [d]	epitope density	yield [%]	activity[a] [%]
1	**1a**	1000/1	**3a**, **4b**, **5a**	2	3.4	100	–
2	**1a**	3000/1	**3a**, **4a**, **5b**	2	9.4	100	–
3	**1a**	4000/1	**2a**, **3a**, **4a**	2	9.9	100	–
4	**1a**	3000/1	**2b**, **3a**, **4a**	1	4.1	49	–
				2	4.8	8.3	–
5	**1a**	3000/1	**2b**, **3a**, **4c**	2	0.5	100	–
				4	1.0	80	–
6	**1a**	1000/1	**2c**, **3a**, **4d**	3	0.0	n.d.[b]	–
7	**1b**	100/1	**2c**, **3a**, **4d**	2	0.0	n.d.[b]	88
8	**1b**	100/1	**3a**, **4d**, **5c**	2	0.0	n.d.[b]	95
9	**1b**	1000/1	**2c**, **3b**, **4a**	1	3.0	75	62
10	**1c**	1000/1	**2b**, **4a**, **5c**	1	3.5	11	93
11	**1c**	2000/1	**2c**, **4a**, **5c**	1	1.0	67	157

[a] Relative to the original activity of the native enzyme. [b] Not determined.

For reactions of **1a** with isonitriles **4a**–**c** (Table 2, entries 1–5) an average epitope density of about 1–10 was obtained. This is in the range for optimal epitope densities of bioconjugates.[18] The Ugi reactions with BSA **1a** as the carboxylic acid component (Table 2, entries 1, 2) and those with BSA **1a** as the amine component (Table 2, entries 3–5) afforded comparable results. However, in all cases a deposition of denaturated protein appeared after three to four days due to probable cross linking of the proteins through an Ugi reaction. This was significant for the conjugation of BSA and **4a** with rhodamine B **2b** as the carbonyl component (Table 2, entry 4). After two days of reaction time, only 8.3% of the respective conjugate was obtained without any improvement of the average epitope density. In contrast, the water soluble isonitrile **4c** (Table 2, entry 5) did not show significant denaturation of the protein even after four days. No formation of conjugates by Ugi reaction could be detected for the reactive water soluble isonitrile **4d** (Table 2, entries 6–8). Neither with BSA **1a** nor with HRP **1b** could any conjugates be detected by mass spectrometry. Thus, the determination of the protein yield was not performed in these cases. The failure of the conjugation in this case can be explained by a significantly higher reactivity of **4d** relative to **4c** which leads to a rather unselective reaction of the isonitrile with the protein. This was also evident from the depletion of the enzymatic activity of **1b** (Table 2, entries 7, 8) without any formation of conjugates by an Ugi reaction. Similar experiments for Ugi reactions of bovine insulin, which showed a better resolution in MALDI-TOF MS than BSA or HRP, revealed that adducts from protein and isonitrile were initially formed in a fast reaction followed by slow hydrolysis during three to four days. Reaction of HRP as the amine or the carbonyl component with isonitrile **4a** (Table 2, entries 9–11) afforded the respective conjugates with average epitope densities of 1–3 in 11–75% yield. Although the reactivity of **4a** and **4d** can be judged as almost equal, the Ugi reaction of **4a** is heterogeneous and thus, proceeds without any unselective blocking of the functional groups of the protein. The retained enzymatic activity of the conjugates with HRP (Table 2, entries 9, 10) was found to be in the range of 62–95% of the initial activity. Thus, the Ugi reaction did not cause any dramatic loss in enzymatic activity. Furthermore, in one case (Table 2, entry 11) the conjugation even resulted in an increase of the enzymatic activity during the Ugi reaction.

In summary, the examples presented here show that Ugi reactions are well suited for the preparation of bioconjugates with up to three components in an exactly defined ratio, provided that careful optimisation of the reaction conditions is done.

Experimental Section

Reaction no. 3 from Table 2: An aqueous solution of acetone (5%, 89.6 μL) and 2 drops of **4a** were added to a solution of BSA (**1a**, M = 66431 Da, 1.0 mg, 15 nmol) and **2a**[11] (19.1 mg, 60 mmol) in 0.1M phosphate buffer (3 mL, pH 7.5) at 25 °C. The mixture was set aside for 2 d and shaken occasionally. Aliquots (500 μL) were withdrawn, centrifuged, and dialysed with water. The molecular weight of the conjugate was determined by MALDI-TOF MS: $M = 70975$ Da; average epitope density = 9.9.

Reaction no. 10 from Table 2: 2 drops of **4a** were added to a solution of HRP freshly oxidized with $NaIO_4$[10] **1c** ($M = 43183$ Da, 1.8 mg, 45 nmol), **2b** (21.6 mg, 45 mmol), and **5c** (14.8 mg, 45 mmol) in 0.1M phosphate buffer (4 mL, pH 7.5) at 25 °C. The mixture was set aside for 1 d and shaken occasionally, centrifuged, and dialyzed with 0.01M phosphate buffer pH 7.5. The amount of protein was determined by measuring the extinction at 280 nm: Yield 4.8 nmol (11%); remaining enzymatic activity of the conjugate(ABTS test[17]) 93%. The molecular weight of the conjugate was determined by MALDI-TOF MS: $M = 46118$ Da; average epitope density was 3.4. The amount of **2b** in the conjugate was determined

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photometrically as 3.5 by measuring the extinction at 575 nm. The biotin density was determined to be 3.3 by a HABA – avidine test.[16]

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μ_4-Peroxo versus Bis(μ_2-Hydroxo) Cores in Structurally Analogous Tetracopper(II) Complexes**

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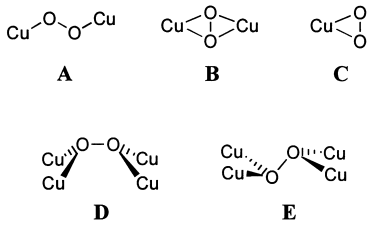
In biological systems, nature often uses the combined redox power of several adjacent metal ions for mediating multi-electron redox transformations. In particular, oligonuclear copper enzymes play a pivotal role in the reversible binding and in the activation of O_2 for oxidation and oxygenation reactions, respectively,[1]—well known representatives include the O_2 carrier protein hemocyanin[2] as well as the enzymes catechol oxidase and tyrosinase.[3] In view of the interest in the use of O_2 in catalytic oxidation reactions, extensive research has been devoted to the search of stable copper – dioxygen adducts[4, 5] to gain insight into the binding of O_2 at oligonuclear copper sites and to understand the function of such metalloenzymes.[6] In this context, structural investigations of copper – peroxo systems are of prime importance.

To date, copper – dioxygen adducts characterized by X-ray crystallography are a set comprised of a complex with *trans*-μ-1,2-peroxo bridge (type **A**),[7] two model complexes for the O_2-binding protein hemocyanin that feature a μ-η^2:η^2-peroxo group (**B**),[5, 8] and a mononuclear η^2-superoxo copper(II) compound (**C**).[9] In addition, a μ_4-peroxo coordination mode



unique in copper chemistry, in which a peroxo group spans four copper(II) ions (**D**), has been described by Krebs et al.[10] Herein, we report a novel example of such unusual μ_4-peroxo coordination (**E**), as well as the X-ray crystallographic characterization of a structurally analogous complex in which the O–O linkage is formally cleaved and replaced by two OH units, while at the same time the overall tetranuclear framework is fully conserved.

The new copper complexes are based on a multidentate pyrazolate ligand L^-.[11] Ligands of this type have proven suitable to hold two metal ions in close proximity and to therefore enable cooperative action of the two metal centers.[12] The metal – metal separation can be tuned by the lengths of the chelating side arms attached to the heterocycle: In complexes of L^- bearing "short" side arms, long metal – metal distances are enforced and small ions like OH^- are

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