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Benzylprotected aromatic phosphonic acids for anchoring peptides on titanium

Jörg Auernheimer and Horst Kessler*

Department Chemie, Lehrstuhl II f. Organische Chemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany

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Abstract—The development of biocompatible coatings is an ongoing issue. Mimicking the physiological adhesion process of osteoblasts to the extracellular matrix improves cell adhesion of osteoblasts in vitro and results in improved and earlier osseous integration of implants in vivo. Titanium, an often used material in implant surgery, can be easily coated by peptides bearing phosphonic acid groups. We report here, the synthesis of benzyl protected phosphonic acids suitable for solid-phase peptide synthesis (SPPS), which can be easily deprotected with TFA.

Osteo-integration of implants is known to be a biological process that occurs by formation of new peri-implant bone in direct contact with the implant surface.[1] Numerous experimental studies have shown that surface modifications can enhance bone/implant contact in terms of both velocity and intensity of bone formation. Accelerated and increased bone contact to the implant surface could be achieved by surface modifications, such as coating with hydroxylapatite,[2] but more advanced improvements of surfaces can be achieved by coating with cyclic RGD peptides[3] of the structure cyclo (-RGDfK-) in which lysine allows coupling of the peptide to anchors.[4] These cyclic peptides specifically bind to αvβ3 and αvβ5 integrins.[5] The αvβ3 integrin is found in focal contacts and leads to spreading and migration of cells onto vitronectin.[6] Adhesion studies elucidated binding specificity of the cyclic RGD-peptides towards osteo-progenitor cells and osteoblasts of different species and in vivo studies confirmed the concept.[7]

For anchoring peptides in a simple way on titanium, which is often used in implant surgery, phosphonic acids can be used. These coatings are extremely stable. Such modified surfaces can be sterilized with γ-irradiation or treated with nitric acid for repassivation.[8] For an introduction of the phosphonic moiety into peptides during solid-phase peptide synthesis (SPPS), the phosphonic acid has to be protected. In the past, we used diethyl-phosphonopropionic acid,[8] but the deprotection of the ethylesters with trimethyl-silyl bromide (TMSBr) worked only with HBr-free TMSBr and caused various side reactions, which led to a very low yield of the desired product. For this reasons, a new protecting group was introduced and the phosphonate building block should bear a branching unit although, as four phosphonic acid groups are needed for a tight anchoring of the peptide to the surface. The second demand was met by a building block used in an artificial RGD-receptor,[9] but the phosphonic acids were still protected as the inappropriate methylesters, which also required TMSBr for deprotection. Our investigations led to the benzyl-protected bisphosphonomethyl benzoic acid **4**, which can be cleaved during the deprotection of the peptide with TFA.

Compound **4** is accessible in a three-step synthesis starting from methyl 3,5-dimethylbenzoate **1** that was brominated in a Wohl–Ziegler bromination. In the following Michaelis–Arbuzov rearrangement with tribenzylphosphite,[10] it is crucial to remove the developing benzylbromide carefully, because it can react with the tribenzylphosphite in the same way. A good removal was achieved by applying a high vacuum of about 0.1 mbar and placing the flask in an oil bath at about

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* Corresponding author. Tel.: +49 89 289 13300; fax: +49 89 289 13210; e-mail: horst.kessler@ch.tum.de

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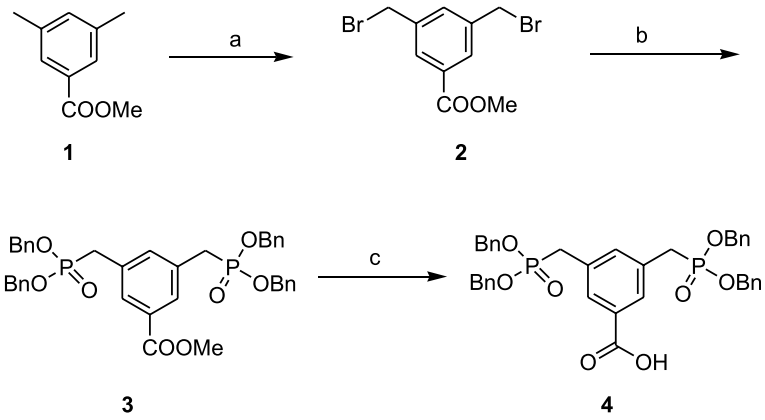


Scheme 1. Synthesis of the benzylprotected anchor building block. Reagents and conditions: (a) 2 equiv NBS, cat. $(PhCOO)_2$, CCl_4 (34%); (b) $(BnO)_3P$ (55%); (c) 1.5 equiv LiOH, $MeOH/H_2O$ (2:1) (96%).

200 °C. The methyl ester of the resulting compound **3** was saponificated with lithium hydroxide to get the benzylprotected bisphosphonic acid **4** (Scheme 1).[11]

A set of linkers, which differ in length and amount of phosphono groups, was synthesized by SPPS using TCP-resin applying standard Fmoc-strategy.[12] These linkers were coupled with the partially protected cyclic peptide cyclo(-R(Pbf)GD(OtBu)fK-)[4] in solution.[13] After the workup, the protecting groups, both on the peptide (Pbf, OtBu) and on the anchor, were cleaved with 95 % TFA, triisopropylsilane and water during 3 h. After RP-HPLC purification,[14] the peptide-linker conjugates were obtained in good yields (Table 1, Scheme 2).

The properties of the conjugates to stimulate cell adhesion on titanium were elucidated with MC3T3-E1 mouse osteoblasts.[15] Therefore, the conjugates were attached to titanium discs (Ti6Al4V, ∅ 1 cm) out of PBS and cells were seeded onto the discs. After 1 h, the number of adherent cells was detected.[16] As shown in Figure 1 conjugates with the new anchor can stimulate the cell adhesion as well as conjugate **10**, known from the literature,[8] if the spacer between the anchor group and the integrin ligand is at least three ε-aminohexanoic acids long.

Table 1. List of synthesized conjugates

Compound	Structure
5	Cyclo(-RGDfK-)-Ahx-Ahx-K-$BPMB_2$
6	Cyclo(-RGDfK-)-Ahx-Ahx-Ahx-K-$BPMB_2$
7	Cyclo(-RGDfK-)-Ahx-Ahx-Ahx-Ahx-K-$BPMB_2$
8	Cyclo(-RGDfK-)-Ahx-Ahx-Ahx-K-(K-$BPMB_2$)$_2$
9	Cyclo(-RβADfK-)-Ahx-Ahx-K-$BPMB_2$
10[8]	Cyclo(-RGDfK-)-Ahx-Ahx-Ahx-K-(K-phosphonopropionyl$_2$)$_2$

BPMB, 3,5-bisphosphonomethyl-benzoyl.

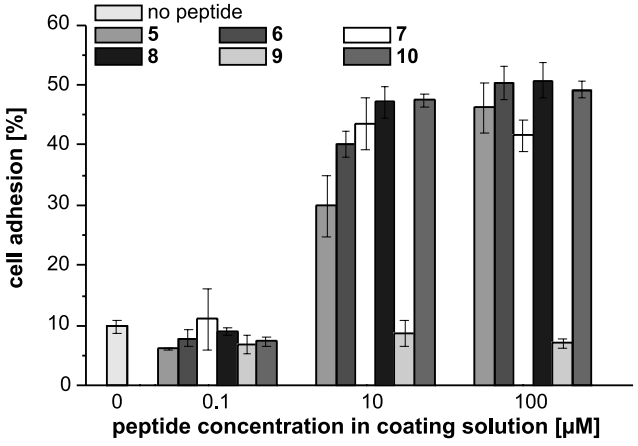
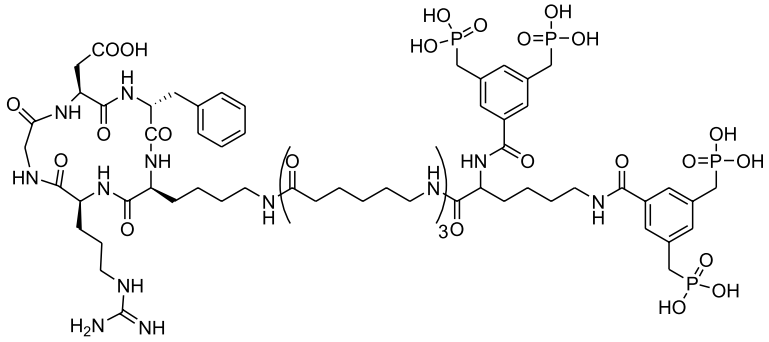


Figure 1. Adhesion of MC3T3 E1 mouse osteoblasts on RGD and RβAD (**9**, as negative control) coated titanium discs. Serum-free media were used for the assay. Conjugate **10**[8] was taken as reference. The numbering is referred to Table 1.



Scheme 2. Structure of cyclic RGD peptide with new phosphonic acid anchors (cyclo(-RGDfK-)-Ahx-Ahx-Ahx-K-$BPMB_2$ **6**).

Furthermore, the cell adhesion is specific to the RGD-peptide and not to the anchor, because the control conjugate **9**, containing the RβAD-sequence, showed no enhanced cell adhesion. Another benefit of the new anchor building block is that only one lysine is required for branching. A second lysine generation, leading to an octameric anchor, showed no effect on cell adhesion.

In conclusion, the synthesis of a benzylprotected bisphosphonic acid was developed. This allows the synthesis of peptides for anchoring on titanium, with standard Fmoc-strategy. The cleavage of benzylesters occurs under the usual deprotection conditions with TFA.

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11. ESI-MS m/z = 671.3 $[m+H]^+$, 693.3 $[m+Na]^+$, 1340.9 $[2m+H]^+$, 1363.0 $[2m+Na]^+$. ^1H NMR (DMSO, 500 MHz): δ (ppm) = 7.80 (2H, s), 7.26 (1H, s), 7.31–7.27 (20H, m), 4.94 (8H, m), 3.37 (4H, dd, J = 5.6, 21.6 Hz).
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13. 1 equiv anchor, 1.1 equiv HOAt, 0.97 equiv HATU, and 10 equiv collidine in 2 mL DMF activation for 2 h followed by addition of 1 equiv cyclo(-R(Pbf)GD(Ot-Bu)fK-). Precipitation in water after 24 h.
14. Colum: YMC-ODS-A 120 5-C18 (5 μm, 250 mm × 20 mm); gradient: 10–60% MeCN in H_2O in 30 min. Both with 0.1% TFA.
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